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Human herpesvirus 6 in multiple sclerosis and encephalitis

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the Small Lecture Hall, Haartman Institute, Haartmaninkatu 3, Helsinki on 23 January 2009, at 12 noon.

Helsinki 2008

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ISBN 978-952-92-4984-8 (paperback)
ISBN 978-952-10-5197-5 (PDF)

http://ethesis.helsinki.fi

Tikkurilan Paino Oy
Vantaa 2008

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List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I **VIRTANEN JO, ZABRISKIE JB, SIRÉN V, FRIEDMAN JE, LYONS MJ, EDGAR M, VAHERI A, KOSKINIEMI M.** *Co-localization of human herpesvirus 6 and tissue plasminogen activator in multiple sclerosis brain tissue. Med Sci Monit 2005;11:BR84-87*

II **VIRTANEN JO, FÄRKKILÄ M, MULTANEN J, UOTILA L, JÄÄSKELÄINEN AJ, VAHERI A, KOSKINIEMI M.** *Evidence for HHV-6 variant A antibodies in multiple sclerosis: diagnostic and therapeutic implications. J Neurovirol 2007;13:347-352*

III **VIRTANEN JO, UOTILA L, FÄRKKILÄ M, VAHERI A, KOSKINIEMI M.** *Human herpesvirus 6 specific oligoclonal IgG bands in multiple sclerosis. Submitted*

IV **VIRTANEN JO, HERRGÅRD E, VALMARI P, AHLQVIST J, FOGDELL-HAHN A, VAHERI A, KOSKINIEMI M.** *Confirmed primary HHV-6 infection in children with suspected encephalitis. Neuropediatrics 2007;38:292-297*

Abbreviations

AAV – *adeno-associated virus*
AbI – *antibody index*
AIDS – *acquired immunodeficiency syndrome*
BBB – *blood brain barrier*
BCIP – *5-Bromo-4-chloro-3-inodyl-phosphate*
CDMS – *clinically definite MS*
CFS – *chronic fatique syndrome*
CMV – *cytomegalovirus*
CNS – *central nervous system*
CPMS – *clinically possible MS*
CSF – *cerebrospinal fluid*
DR – *terminal direct repeat*
EAE – *experimental autoimmune encephalomyelitis*
EBV – *Epstein–Barr virus*
ES – *exanthema subitum*
gB, gH, gL, gM, gQ – *glycoprotein B, H, L, M, Q*
HHV – *human herpesvirus*
HIV – *human immunodeficiency virus*
HSV – *herpes simplex virus*
IE – *immediate early*
IEF – *isoelectric focusing*
Ig – *immunoglobulin*
ITAP – *intrathecal antibody production*
LTP – *large tegument protein*
MBP – *myelin basic protein*
MCP – *membrane cofactor protein*
MMPs – *matrix metalloproteinases*
MS – *multiple sclerosis*
NAWM – *normal appearing white matter*
NBT – *4-Nitro blue tetrazolium chloride*
OCB – *oligoclonal band*
OG – *oligodendrocyte*
OND – *other neurological disease*
ORF – *open reading frame*
RDA – *representational difference analysis*
ssRNA – *single stranded RNA*
Tc1 – *cytotoxic T cell 1*
Th1, Th2 – *T-helper cell 1, 2*
tPA – *tissue plasminogen activator*
VZV – *varicella-zoster virus*

Abstract

Human herpesvirus 6 (HHV-6) was identified from patients with HIV and lymphoproliferative diseases in 1986. It is a β-herpesvirus and is divided into two subgroups, variants A and B. HHV-6 variant B is the cause of *exanthema subitum*, while variant A has not yet definitely proven to cause any disease. HHV-6, especially variant A, is a highly neurotropic virus and has been associated with many diseases of the central nervous system (CNS) such as encephalitis and multiple sclerosis (MS).

The present studies were aimed to elucidate the role of HHV-6 and its two variants in neurological infections. Special attention was given to study the possible role of HHV-6 in the pathogenesis of MS.

We studied the expression of HHV-6 antigens using immunohistochemistry in brain autopsy samples from patients with MS and controls. HHV-6 antigen was identified in 70% of MS specimens whereas 30% of control specimens expressed HHV-6 antigen. In addition, HHV-6 antigen was associated with elevated expression of tissue plasminogen activator (tPA), an enzyme taking part in both neuronal development and degeneration.

In order to study the role of HHV-6 in MS further, serum and cerebrospinal fluid (CSF) samples were collected from patients with MS and patients with other neurological diseases (OND) from patients visiting Helsinki University Central Hospital Neurological Outpatient Clinic during the years 2003 and 2004. A total of 27 patients with clinically definite MS (CDMS) and 19 patients with clinically possible MS (CPMS) and age- and gender-matched control patients with OND were included. In addition, we studied 53 children with suspected encephalitis. Samples were consecutively collected from the first and last quarter of the year 1995, from the sample series sent to the Department of Virology, University of Helsinki, for suspected viral encephalitis.

We developed an immunofluorescence IgG-avidity assay for the detection of primary HHV-6A and HHV-6B infection. For HHV-6B antibodies, no differences were observed between patients with MS and OND.

For HHV-6A both seroprevalence and mean titers were significantly higher in MS compared to OND. HHV-6A low-avidity IgG antibodies, suggestive of primary infection, were found in serum of two, three and one patient with CDMS, CPMS and OND, respectively. From pediatric patients with suspected encephalitis, six serum samples (11.3%) contained low-avidity antibodies, indicating a temporal association between HHV-6A infection and onset of encephalitis. From these six samples one had HHV-6 DNA in serum and two in CSF.

Three out of 26 patients with CDMS and four out of 19 patients with CPMS had HHV-6 antibodies in their CSF compared to none of the patients with OND ($p = 0.06$ and $p = 0.01$, respectively). When HHV-6 antibodies were compared to the total IgG in serum and CSF, two patients with CDMS and three patients with CPMS appeared to have specific intrathecal synthesis of HHV-6A antibodies. In addition, oligoclonal bands (OCB) were observed in the CSF of five out of nine MS patients tested, and in two the OCBs reacted specifically with HHV-6 antigen, which is a novel finding. These results indicate HHV-6 specific antibody production in the CNS and suggest that there is a subset of MS patients with an active or chronic HHV-6A infection in the CNS that might be involved in the pathogenesis of MS.

Our studies suggest that HHV-6 is an important causative or associated virus in some neurological infections, such as encephalitis and it might contribute to the development of MS, at least in some cases. In conclusion, HHV-6 is a neurotropic virus that should be taken into consideration when studying acute and chronic CNS diseases of unknown origin.

Tiivistelmä

(Summary in Finnish)

Ihmisen herpesvirus 6 (HHV-6) eristettiin HIV-positiivisen lymfoproliferatiivisista sairauksista kärsivien potilaiden verestä vuonna 1986. HHV-6 kuuluu β-herpesvirusten ryhmään ja esiintyy kahtena eri varianttina, A ja B. HHV-6 variantti B:n on todettu aiheuttavan vauvarokkoa. HHV-6 variantti A:n ei ole kiistatta todistettu aiheuttavan mitään tautia, vaikkakin hyvin hermostohakuisena viruksena se on yhdistetty keskushermoston sairauksiin kuten aivotulehdukseen ja aivojen pesäkekovettumatautiin (multippeli skleroosi, MS-tauti).

Väitöskirjatutkimuksen tarkoituksena oli tutkia HHV-6:n ja sen kahden eri variantin aiheuttamia keskushermostoinfektioita. Väitöskirjan keskeisenä tavoitteena oli ennenkaikkea tutkia myös HHV-6:n osuutta MS-taudin synnyssä ja kehityksessä.

Tutkimme HHV-6-proteiinien ilmenemistä MS-tautia sairastaneiden potilaiden ruumiinavauksen yhteydessä saaduista aivokudosnäytteistä ja vertasimme tuloksia kontrollipotilaiden aivokudoslöydöksiin. HHV-6 antigeeniä todettiin 70 %:lla MS-tautia sairastaneiden potilaiden aivokudoksessa, kun taas verrokeilla vastaava luku oli 30 %. Lisäksi havaitsimme, että HHV-6:n antigeenin ilmeneminen liittyi kohonneeseen kudostyypin plasminogeenin aktivaattorin (t-PA) tasoon.

Tutkiaksemme tarkemmin HHV-6:n osallisuutta MS-taudin synnyssä ja kehityksessä keräsimme seerumi- ja likvorinäytteitä MS-tautia sairastavilta potilailta sekä verrokkipotilailta. Näytteet kerättiin Helsingin yliopistollisen keskussairaalan neurologian poliklinikalla vuosina 2003–2004. Tutkimusaineistossa 27 potilaalla todettiin varma ja 19 mahdollinen MS-diagnoosi. Potilaille valittiin ikään ja sukupuoleen perustuen vastaavat verrokit potilaista, jotka sairastivat jotain muuta hermoston sairautta. Sisällytimme aineistoon myös 53 aivotulehdusepäilyn takia tutkimuksiin lähetetyn lapsipotilaan näytteet. Seerumit kerättiin arkistomateriaalista, jotka oli lähetetty Helsingin yliopiston virologian osastolle Haartman-

instituuttiin, vuoden 1995 ensimmäisellä ja viimeisellä neljänneksellä.

Kehitimme immunofluoresenssiin perustuvan IgG-aviditeettitestin HHV-6 variantti A:n ja variantti B:n primaari-infektioiden toteamiseksi seerumista. HHV-6B tyypin vasta-aineissa ei todettu mitään eroa eri ryhmien välillä, mutta HHV-6A tyypin vasta-aineita löytyi useammin ja suurempina pitoisuuksina MS-tautia sairastavien potilaiden seerumissa verrattuna kontrollipotilaisiin. Primaari-infektioon viittaavia matala-avidisia vasta-aineita löydettiin kahdelta varmalta ja kolmelta mahdolliselta MS-potilaalta ja yhdeltä muuta hermoston sairautta sairastavalta potilaalta. Kuudella 53 aivotulehdusepäilylapsesta (11.3 %) havaittiin matala-avidisia vasta-aineita viitaten primaari-infektioon. Yhdellä oli HHV-6 DNA:ta seerumissa ja kahdella likvorissa.

Likvorista löytyi HHV-6 vasta-aineita kolmelta kliinisesti varmalta MS-potilaalta ja neljältä mahdolliselta, kun taas kontrolliryhmässä ei kenelläkään ollut likvorissa HHV-6 vasta-aineita ($p = 0.06$ ja $p = 0.01$). Kahdella kolmesta varmasta ja kolmella neljästä mahdollisesta MS-potilaasta vasta-aineiden tuotto näytti olevan aivoperäistä, intratekaalista, kun verrattiin HHV-6 spesifin vasta-aineen tasoa seerumin ja likvorin IgG:n kokonaismäärään. Tämän lisäksi viidellä yhdeksästä tutkitusta MS-potilaasta todettiin likvorissa oligoklonaalisia vyöhykkeitä ja kahdella potilaalla nämä vyöhykkeet reagoivat spesifisesti HHV-6 antigeenin kanssa. Tällaista reaktiota ei MS-potilailla ole aikaisemmin osoitettu. Tulokset viittaavat, että osalla MS-potilaista saattaa olla aktiivinen tai krooninen HHV-6 infektio vaikuttaen MS-taudin syntyyn ja kehitykseen.

Väitöskirjan tutkimustulokset viittaavat siihen, että HHV-6 on etiologinen tai myötävaikuttaja tekijä joissakin keskushermoston infektioissa ja erityisesti se näyttäisi liittyvän MS-tautiin, vaikkakin sen rooli taudin synnyssä ja kehityksessä vaatii lisäselvittelyä. HHV-6 on syytä ottaa huomioon tutkittaessa etiologialtaan tuntemattomia keskushermoston sairauksia.

Review of the literature

Herpesviridae

Herpesviridae is a large family of double-stranded DNA viruses containing more than one hundred recognized species, although it is likely that the identified herpesviruses thus far represents only a small fraction of their true number. Herpesviruses infecting a wide range of vertebrates and at least one herpesvirus infecting an invertebrate have been identified (Davison, 2002). Herpesviruses have a strict host specificity suggesting long co-evolution with their hosts. The name of the family is derived from the Greek word *herpein* (= to creep) and refers to the latency and reoccurrence of the infections caused typically by herpesviruses. The *Herpesviridae* family is divided into three subfamilies: *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae*.

Human herpesviruses

Eight different herpesviruses infecting humans, abbreviated as HHV-1 to HHV-8, have been identified to date. It has been suggested, but not formally accepted, that HHV-6 variants A and B could be considered two separate viruses (Komaroff *et al.*, 2006), increasing the number of the members to nine. The first five and the last member of human herpesviruses are commonly known by their alternative names, while sixth and seventh members are known as human herpesvirus 6 and 7, respectively (Table 1). The members of *alphaherpesvirinae* causes typically skin blisters. The members of *gammaherpesvirinae* have the potential to malignant transformation of the cell and typically cause lymphomas and Kaposi's sarcoma. Cytomegalovirus (CMV) and HHV-6 variant B, members of the *betaherpesvirinae*, cause mononucleosis and *exanthema subitum* (ES), respectively. The diseases caused by other two members of *betaherpesvirinae*, HHV-6 variant A and HHV-7, are not known, but some cases of ES might be caused by HHV-7 (Bruns *et al.*, 2000), and HHV-6A has been associated with neurological infections (Boutolleau *et al.*, 2006).

Table 1. Human herpesviruses.

		disease/symptoms	subfamily/genus
HHV-1	Herpes simplex virus 1 (HSV-1)	oral/genital herpes (predominantly orofacial)	*Alphaherpesvirinae/ Simplexvirus*
HHV-2	Herpes simplex virus 2 (HSV-2)	oral/genital herpes (predominantly genital)	*Alphaherpesvirinae/ Simplexvirus*
HHV-3	Varicella-zoster virus (VZV)	chickenpox, shingles	*Alphaherpesvirinae/ Varicellovirus*
HHV-4	Epstein-Barr virus (EBV)	mononucleosis, lymphomas	*Gammaherpesvirinae/ Lymphocryptovirus*
HHV-5	Cytomegalovirus (CMV)	mononucleosis, retinitis	*Betaherpesvirinae/ Cytomegalovirus*
HHV-6A	Human herpesvirus 6 variant A	No clear disease associations, CNS symptoms?, multiple sclerosis?	*Betaherpesvirinae/ Roseolovirus*
HHV-6B	Human herpesvirus 6 variant B	*exanthema subitum*, CNS symptoms?, epilepsy?	*Betaherpesvirinae/ Roseolovirus*
HHV-7	Human herpesvirus 7	roughly similar symptoms as HHV-6B	*Betaherpesvirinae/ Roseolovirus*
HHV-8	Kaposi's sarcoma associated herpesvirus (KSHV)	Kaposi's sarcoma, lymphomas	*Gammaherpesvirinae/ Rhadinovirus*

Human herpesvirus 6

HHV-6, a member of the *Roseolovirus* genus within the *Betaherpesvirinae* subfamily, and its two variants A and B are further discussed in the following sections.

History

Human herpes virus 6 (HHV-6) was for the first time isolated from patients with HIV and lymphoproliferative diseases in 1986 (Salahuddin *et al.*, 1986). Initially, the virus was named human B-lymphotropic virus (HBLV), due to its apparent tropism for B-lymphocytes. Soon it became evident that the virus had a much wider tropism and it actually preferentially infected T-lymphocytes rather than B-lymphocytes, and was consequentially renamed human herpesvirus 6. The second major breakthrough in the natural history of the HHV-6 occurred in 1988, when a Japanese group showed by virus isolation and seroconversion that HHV-6 is the cause of *exanthema subitum* (roseola infantum, sixth disease, ES) (Yamanishi *et al.*, 1988). In the late 1980s and

early 1990s, a number of new virus strains were isolated and they seemed to fall into two classes (Schirmer *et al.*, 1991), and variants A and B (HHV-6A and HHV-6B) were established. They differed in their genome and in their growth properties and cell tropism as well as in immunology (Ablashi *et al.*, 1991). The initial isolate appeared to be variant A, while the variant behind ES was HHV-6B (Schirmer *et al.*, 1991). The primary infection was shown to be caused by variant B in the majority of the population in the U.S. (Dewhurst *et al.*, 1993a).

Since the early stages of HHV-6 research, based on the patients from which HHV-6 (strain GS, variant A) was isolated, it was obvious to look for associations between HHV-6 and HIV and acquired immunodeficiency syndrome (AIDS). Several articles described high levels of antibodies to HHV-6 in patients with HIV infection (Brown *et al.*, 1988; Fox *et al.*, 1988; Krueger *et al.*, 1988) and it was shown that HHV-6 can double-infect cells after prior HIV infection (Agut *et al.*, 1988; Agut *et al.*, 1989; Lusso *et al.*, 1989) and two pioneers, co-discoverer of HIV Dr. Robert Gallo, and Dr. Paulo Lusso suggested an association between HHV-6 with AIDS (Lusso and Gallo, 1994; Lusso and Gallo, 1995).

Already at the beginning it was shown that HHV-6 is a neurotropic virus and can cause severe CNS disease (Wakefield *et al.*, 1988; Ishiguro *et al.*, 1990; Asano *et al.*, 1992; Merelli *et al.*, 1992). The first publication reporting HHV-6 antibodies and DNA in multiple sclerosis was published soon after (Sola *et al.*, 1993) and an association was suggested two years later (Challoner *et al.*, 1995). Active HHV-6 infection was also associated with chronic fatigue syndrome (CFS) (Buchwald *et al.*, 1990; Josephs *et al.*, 1991; Buchwald *et al.*, 1992).

The problem in unraveling the viral pathogenesis of HHV-6 is the commensalism and the ubiquitous nature of the virus. The latency complicates the detection of the active infection, not mention the recent discovery that HHV-6 can integrate into the host chromosome thus interfering molecular diagnosis. Although much improvement has been made during the latest years with respect to the molecular and cellular biology of the HHV-6 infection, much further investigation is needed.

Structure and genome

HHV-6 shares a common structure with other herpesviruses. It is composed of three main structural elements; nucleocapsid, tegument and envelope. The nucleocapsid containing the viral genome has an icosahedral symmetry made up of 162 capsomers (Biberfeld *et al.*, 1987). The tegument layer covers the nucleocapsid and is composed of a protein mixture. Finally the viral particle is covered by an envelope, in which the viral glycoproteins are embedded (Figure 1).

The genome of HHV-6 is linear double-stranded DNA molecule. The size of the DNA molecule is 160 to 162 kb depending on the variant and isolate. The genome consists of unique (U) region, which is 143 to 145 kb in size and is flanked by terminal direct repeats (DR). DR regions are approximately 8 to 9 kb. Three additional repeat regions, R1, R2 and R3 truncate the U region in the immediate early A (IE-A) region (Figure 2). Each end of the DRs is composed of telomeric repeats $(GGGTTA)_n$ (Thomson *et al.*, 1994). Telomeric repeats have been postulated to have a role in DNA replication and maintenance of the viral genome in latently

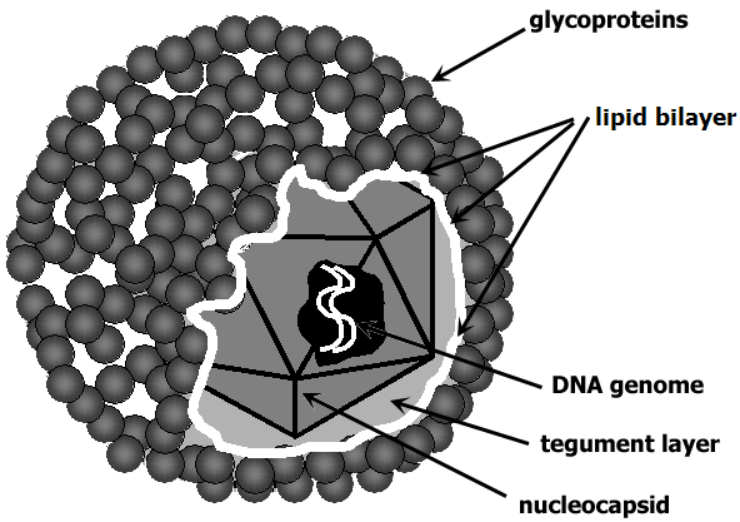

Figure 1. Schematic presentation of typical herpesvirus structure. Double stranded DNA genome is packed inside the protein coat called nucleocapsid. The tegument layer covers the nucleocapsid. The outer layer, envelope, consists of lipids and glycoproteins.

infected cells (Gompels and Macaulay, 1995; Gompels *et al.*, 1995). The genes within the DRs are designated DR1 to DR8 and genes within the U region are called U1 to U100 (Figure 2).

The similarity between variants A and B at the nucleotide level is 90% (Gompels and Macaulay, 1995; Dominguez *et al.*, 1999). The similarity is highest in the central part of the genome and decreases when approaching genomic ends, being lowest at the IE region (Dominguez *et al.*, 1999; Isegawa *et al.*, 1999). Although high variation between variants exists, IE region is highly conserved among HHV-6B isolates (Stanton *et al.*, 2003). Nine open reading frames (ORFs) found in the HHV-6B genome are absent in HHV-6A (Dominguez *et al.*, 1999) and, on the other hand, nine ORFs present in the HHV-6A genome cannot be found in HHV-6B (Gompels *et al.*, 1995).

HHV-6 variants A and B

Although HHV-6B is the cause of ES (Yamanishi *et al.*, 1988), HHV-6A has not been unequivocally shown to cause any disease. These two variants differ in epidemiology, in vitro growth properties, reactivity with monoclonal antibodies, restriction endonuclease mapping and nucleotide and amino acid sequences (Wyatt *et al.*, 1990; Ablashi *et al.*, 1991; Aubin *et al.*, 1991; Schirmer *et al.*, 1991; Chandran *et al.*, 1992; Gompels *et al.*, 1993; Yamamoto *et al.*, 1994; Isegawa *et al.*, 1999). The differences are discussed in detail in the appropriate paragraphs. Some researchers have suggested that these two variants could actually be considered two separate β-herpesviruses (Komaroff *et al.*, 2006). Nevertheless, the International Committee on Taxonomy of Viruses regards HHV-6A and HHV-6B as strains or different isolates, not separate viruses (Büchen-Osmond, 2008).

Cell tropism

Although initially named human B-lymphotropic virus, HHV-6 is predominantly regarded as T-cell tropic. In reality, HHV-6 infects a wide variety of cell types (Ablashi *et al.*, 1987; Ablashi *et al.*, 1989). All isolates including both variants A and B grow in activated peripheral blood mononuclear cells; however, the variants can be distinguished by culture in continuous T-cell lines. HHV-6A

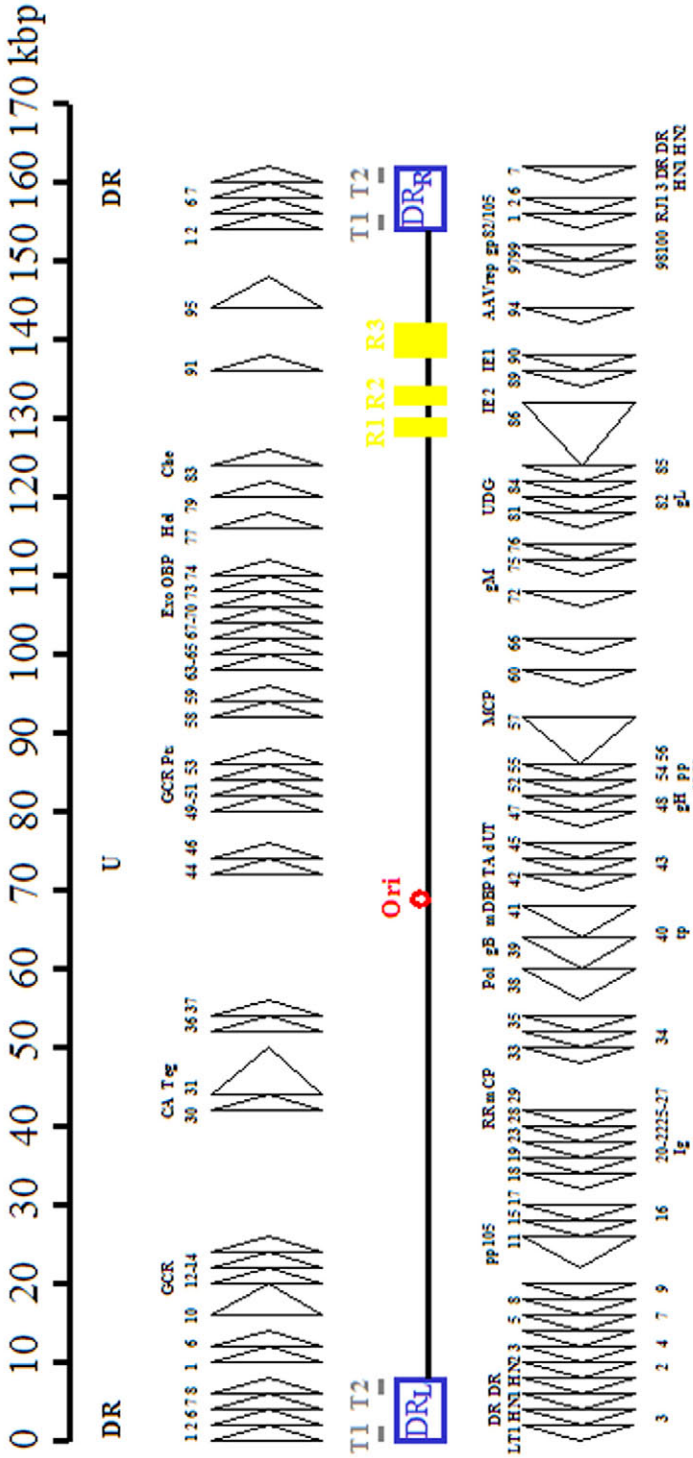

Figure 2. Genome organization of the HHV-6B. Terminal direct repeats (DRs) are boxed and the intermediate repeats are shown as yellow boxes. The origin of replication (ori) is presented as red circle. Protein-coding regions are presented as triangles (note that the size of the triangle is only indicative of the length of the ORF). Abbreviations: GCR, G-protein-coupled receptor; Ig, immunoglobulin superfamily; RR, ribonucleotide reductase; mCP, minor capsid protein; CA, capsid assembly protein; Teg, large tegument protein; Pol, DNA polymerase; tp, transport protein; mDBP, major single-stranded DNA-binding protein; TA, conserved herpesvirus transactivator; dUT, dUTPase; Pts, protease/assembly protein; MCP, major capsid protein; PT, phosphotransferase; Exo, exonuclease; OBP, origin binding protein; Hel, helicase; UDG, uracil-DNA glycosylase; Che, chemokine; AAVrep, adeno-associated virus-2 replication protein homolog. Modified from Isegawa et al. (1999).

infects efficiently HSB-2 cell line, but not MOLT-3 cells. HHV-6B infects MOLT-3 cells, but not HSB-2 cells (Ablashi *et al.*, 1991). However, intervariant variation between different isolates might also exist.

HHV-6 is a neurotropic virus and has been shown to infect primary human fetal astrocytes (He *et al.*, 1996), glioblastoma cells (Ablashi *et al.*, 1989), neuroblastoma cells (Levy *et al.*, 1990) and embryonic glia (Tedder *et al.*, 1987). HHV-6A seems to be more neurotropic than HHV-6B is (Hall *et al.*, 1998; Ahlqvist *et al.*, 2005; De Bolle *et al.*, 2005b). Differential tropism has been observed in astrocytes (Donati *et al.*, 2005; Ahlqvist *et al.*, 2006) and in oligodendrocytes. In astrocytes only HHV-6A is able to complete a full replication cycle (Ahlqvist *et al.*, 2006). HHV-6A is able to form an active infection and latent infection after active phase while HHV-6B forms an abortive infection in the oligodendrocytic cell line MO3.13 (Ahlqvist *et al.*, 2005).

Replication cycle

CD46, or membrane cofactor protein (MCP), is the cellular receptor for HHV-6 (Santoro *et al.*, 1999) (Figure 3). CD46 is present in all nucleated cells (Liszewski *et al.*, 1991) and might partially explain why HHV-6 can infect such a wide variety of human cells and the restriction of permissive species. HHV-6 glycoproteins H, L and Q (gH, gL and gQ) form a complex that associates with CD46 (Mori *et al.*, 2003). gH is the glycoprotein that directly binds to the short consensus repeat domains SCR2 and SCR3 of CD46 (Santoro *et al.*, 2003). Glycoprotein B (gB) is not directly involved in virus-CD46 interaction, but it has been shown to have a role in the fusion event as well (Takeda *et al.*, 1996). gB contains variant-specific epitopes and is a target for neutralizing antibodies (Campadelli-Fiume *et al.*, 1993; Takeda *et al.*, 1996), which might contribute to the different cell tropisms of HHV-6 variants.

After attachment to the cell membrane by glycoprotein-CD46 interaction, the viral envelope fuses with the cell membrane and nucleocapsid enters the cytoplasm. In the fusion both viral cholesterol within viral membrane (Huang *et al.*, 2006) and cellular cholesterol within plasma membrane (Tang *et al.*, 2008) are

required for successful entry into the cell. The transportation mechanisms are not well understood, but it is hypothesized that nucleocapsid is transported in a similar way as HSV-1 (Lycke *et al.*, 1988) and CMV (Ogawa-Goto *et al.*, 2003), *i.e.* by association with the microtubule network. After reaching the nuclear pore complex the viral DNA is released inside the nucleus, where the virus uses the cellular transcription and translation machinery to produce viral proteins. Immediate early (IE) or α proteins are produced within few hours after the viral enter to the cell, and these proteins regulate the transcription of the other genes. Subsequently, IE proteins initiate the transcription of early (E) or β genes. E gene products are mainly involved in DNA metabolism and replication. Late (L) or γ genes, which are either partially or fully dependent on viral DNA replication, are transcribed last. Products of L genes are structural proteins and other proteins that are involved in virion assembly. Interestingly, Øster and Höllsberg (2002) have shown that many of the genes that are E or even L are actually detected as early as one hour after infection. This observation might suggest that HHV-6 genes are leaky or might be controlled by a more complex mechanism. Indeed, in the case of HCMV and HSV the temporal gene expression pattern follows five kinetic classes rather than three. Genes are subdivided into α (immediate early), β1 (early), β2 (early late), γ1 (leaky late) and γ2 (true late) genes (Roizman *et al.*, 2007; Mocarski *et al.*, 2007). In Øster and Höllsberg studies (2002) the HHV-6B genes seemed to fall in six different classes.

DNA replication of the HHV-6 needs seven virally encoded proteins. Replication is initiated by the binding of the origin binding protein (encoded by U73 gene) to the origin of lytic replication (ori-lyt) evoking denaturation of a portion of the circular genome (Dewhurst *et al.*, 1993b; Inoue *et al.*, 1994; Inoue and Pellett, 1995). Three viral proteins, encoded by U43, U74 and U77, form a heterotrimeric helicase/primase complex, which maintains the DNA helix unfolded and provides RNA primers to the lagging-strand synthesis (Nicholas, 1994). Major DNA-binding protein encoded by U41 stabilizes the single-stranded DNA until the second

strand is produced by DNA polymerase encoded by U38 gene (Teo *et al.*, 1991). U27 encodes a protein that binds specifically to DNA polymerase acting as a processivity factor (Zhou *et al.*, 1994; Lin and Ricciardi, 1998). Besides these seven proteins, four additional proteins encoded by U79-80 genes, and alternatively spliced after translation, have been suggested to have a role in viral DNA replication (Taniguchi *et al.*, 2000).

The replication proceeds by rolling-circle replication and concatameric progeny DNA strands are encapsidated by the interaction of the cleavage and packaging proteins with the specific packaging signals *pac1* and *pac2* at the both ends of the DNA molecule (Thomson *et al.*, 1994; Deng and Dewhurst, 1998).

Capsids bud out from the nucleus. From the membrane of the nucleus the capsid acquires

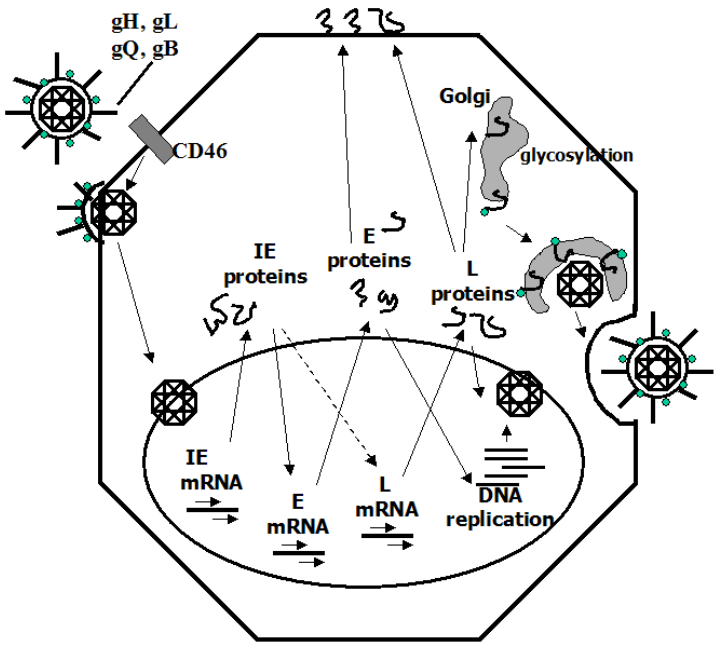

Figure 3. HHV-6 replication cycle. HHV-6 enters the cell using CD46 as a cellular receptor. The genes are transcribed and translated in three kinetic classes, immediate early (IE), early (E) and late (L) genes. HHV-6 egress the cell by endocytosis. The replication cycle events; entry, replication, maturation and egress, are described in the text. Modified from De Bolle et al. (2005a).

a temporal membrane that does not include glycoproteins. In contrast to other herpesviruses HHV-6 glycoproteins are not present on the plasma membrane of an infected cell (Cirone *et al.*, 1994; Torrisi *et al.*, 1999). HHV-6 glycoproteins accumulate either to the Golgi complex (where they are glycosylated) or to anullate lamellae (stack of narrow membranes present in the cytoplasm near the rough endoplasmic reticulum). Capsids acquire their envelope that contains glycoproteins from the Golgi complex or anullate lamellae (Cardinali *et al.*, 1998) and mature virus particles are released from the cell by exocytosis. Recently, it has been suggested that mature viral particles are transferred to the plasma membrane within multivesicular body-like compartments that include small vesicles. Mature virions together with small vesicles are released to the extracellular space by an exosomal pathway (Mori *et al.*, 2008).

Epidemiology

The primary infection by either or both variants occurs during the first three years of life in up to 90% children throughout the world. An initial report on seroprevalence showed a really low (2%) seroprevalence among healthy blood donors and AIDS patients without lymphoma (0%) in U.S. (Salahuddin *et al.*, 1986). Krueger *et al.* (1988) and Ablashi *et al.* (1988) then showed that the seroprevalence was approximately one fourth in the general population when very strict diagnostic criteria was used, but the seroprevalence increased to 63%, if lower and borderline antibody titers were included. Therefore, it is likely that these early conflicting results were due to the subjectivity, sensitivity and interpretation of fluorescence assays, or the virus strain used as the source of the antigen, not to mention geographic variation.

Over 90% newborns have maternal antibodies; these maternal antibodies however, disappear between birth and six months of age (Enders *et al.*, 1990). In some cases maternal antibodies can persist beyond one year thus overestimating the HHV-6 infection rates in young children in seroprevalence studies (Chokephaibulkit *et al.*, 1997). The peak of HHV-6 infection occurs at the age of six to 15 months (Okuno *et al.*, 1989; Enders *et al.*, 1990). A slight increase in the

seropositivity occurs during the first decade of life, but HHV-6 antibody titers show a decline with advancing age (Saxinger *et al.*, 1988; Enders *et al.*, 1990; Cermelli *et al.*, 1992; Parker and Weber, 1993; Baillargeon *et al.*, 2000; Ihira *et al.*, 2002). Overall, HHV-6 is a widespread virus with prevalences ranging from 60-100% in different geographical regions (Linhares *et al.*, 1991; Chua *et al.*, 1996; Nielsen and Vestergaard, 1996; Tolfvenstam *et al.*, 2000; Bhattarakosol *et al.*, 2001). In Africa, HHV-6A infection seems to be more prevalent than HHV-6B infection in infants (M. Bates, University of London, personal communication).

HHV-6 diagnostics

The diagnostics of HHV-6 is a complicated issue. Many features of HHV-6 make the diagnosis laborious and difficult to interpret; including two variants with different pathological properties, latency, integration into the genome and high prevalence among the population (Koch, 2001; Dockrell, 2003; Ward, 2005). Each of the above features should be taken into consideration when assessing diagnostic approaches along with careful evaluation

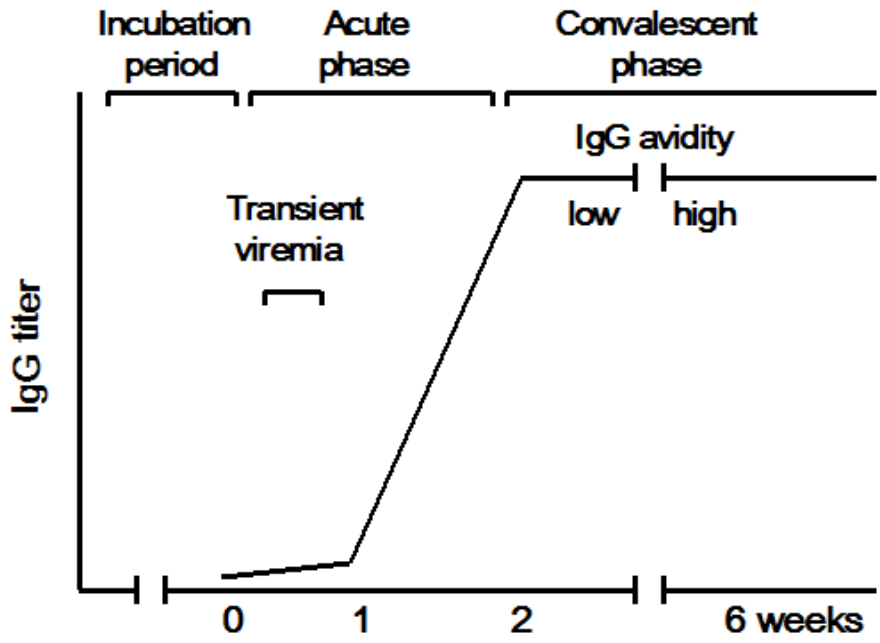

Figure 4. Virological features of primary HHV-6 infection (Ward, 2005).

of the clinical picture. The most common problem in HHV-6 diagnosis is either lack of variant distinction or lack of distinction between primary infection, chronic infection or reactivation. Although reactivation might happen in immunocompromised patients, the clinical significance of the reactivation is not clear (Clark and Griffiths, 2003). Primary infection can be diagnosed either by the presence of DNA in serum during viremia at the acute phase in the absence of IgG antibodies or as the presence of low-avidity IgG antibodies in serum at the late acute or convalescent phase (Figure 4). In the former case, HHV-6 DNA integration into the genome might lead to misdiagnosis (Tanaka-Taya *et al.*, 2004; Clark *et al.*, 2006; Ward *et al.*, 2006). The gold standard to diagnose primary infection is to detect IgG seroconversion between two serum samples.

PCR and molecular diagnostics

Nucleic acid amplification methods, mainly PCR, have been used for detection of HHV-6 DNA in many body fluids as well as in tissues. The clinical significance of a positive viral DNA finding in different body fluids is somewhat controversial. The most common problem in DNA amplification is that one cannot differentiate latent infection from active infection. In the general population there is a low copy number of viral DNA present in blood lymphocytes representing latent infection. Serum and plasma usually do not contain free viral DNA. In the absence of IgG, a high copy number in whole blood or the presence of any viral DNA in serum or plasma have been shown to occur during transient viremia during the acute phase (Figure 4) indicating primary infection (Asano *et al.*, 1989). This was shown by Chiu *et al.* (1998) to be the most reliable method after seroconversion to detect HHV-6 primary infections, although occasionally viral DNA in plasma or a high copy number in whole blood was associated with other infections possibly representing HHV-6 reactivation. The major advantage of PCR is the possibility to distinguish the two variants from each other. Indeed, many variant-specific PCR methods have been developed.

Variant distinction can be made by using either variant-specific primers that selectively amplify only one of the vari-

ants or primers that amplify a fraction from both variants, which are further identified e.g. by the use of variant-specific probes in hybridization (Drobyski *et al.*, 1993; Aubin *et al.*, 1994). Real-time PCR methods have been developed for the quantification of the amount of genomes present in the samples (Safronetz *et al.*, 2003; Boutolleau *et al.*, 2006). In many cases detection of DNA cannot reveal whether the infection is active or latent, although quantification may give indication. The expression of HHV-6 RNA on the other hand is a real marker of active infection and can be detected by the means of reverse transcription PCR (Norton *et al.*, 1999; Yoshikawa *et al.*, 2003; Alvarez-Lafuente *et al.*, 2004; Pradeau *et al.*, 2006). A nested step can be used in PCR to improve sensitivity, but it might produce false-positive results and thus all of the amplified fragments are recommended to be sequenced and therefore the method is too laborious for the routine diagnostics.

Dr. Flamand and collaborators (Flamand *et al.*, 2008) initiated a multicenter study, to evaluate the differences and reliability in different PCR methods used currently. The lack of standardized PCR assay might have led to discordant results in disease associations. The authors conclude that from six different real-time PCRs, three seemed to be most reliable and research and diagnostic laboratories should use similar or identical to those as reference in setting up their own HHV-6 assay.

Multiplex-PCR assays have been developed to detect a variety of human herpesviruses (Read and Kurtz, 1999; Markoulatos *et al.*, 2001) e.g. for screening purposes in CNS infections. Microarray-based technology following multiplex-PCR has been developed to identify different herpesviruses (Jääskeläinen *et al.*, 2006; Jääskeläinen *et al.*, 2008).

Serological diagnostics

Maternal antibodies are present in sera of infants under the age of approximately six months (Enders *et al.*, 1990) and make the use of serology difficult in early childhood. After the disappearance of the maternal antibodies, primary infection in children can be detected reliably by detection of seroconversion. The first serum sample is taken immedi-

ately after the symptoms and is usually negative for IgG. A follow-up sample is taken during the convalescent phase one to two weeks after onset of symptoms and if positive, seroconversion is detected. IgM serology is not a reliable marker of primary infection since it might be positive in reactivations and reinfections and furthermore, it is not always positive in primary infections (Fox *et al.*, 1988; Suga *et al.*, 1992; Salonen *et al.*, 2008).

IgG avidity can be used for the detection of primary infection in IgG-positive samples at the convalescent phase (Hedman *et al.*, 1993). Initially after the primary infection B-cells produce antibodies that have a weak overall binding strength (low avidity) to the antigen. After B-cell maturation the antibodies produced have higher overall binding strength (high avidity). The maturation of B-cells in the case of HHV-6 infection usually takes approximately four to six weeks and thus the diagnostic window for the detection of primary infection from the convalescent serum is wide. The IgG-avidity methodology for the detection of primary HHV-6 infection was introduced in 1993 (Ward *et al.*, 1993b) and shown to be specific for HHV-6 and not to cross-react with other herpesviruses (Ward *et al.*, 1993a). Although it is generally claimed that HHV-6 variants A and B cross-react serologically with each other, some studies have reported different titers and prevalences for these two (Ongradi *et al.*, 1999; Portolani *et al.*, 2006). Portolani *et al.* (2006) were able two show a primary HHV-6A infection in an adult patient with encephalomyelitis using HHV-6A and HHV-6B infected cells, and it was confirmed by variant-specific PCR.

Clinical presentations and associations of HHV-6

Only one disease, ES, has been etiologically proven to be caused by HHV-6. However, many diseases have been associated with the virus and are discussed in the following sections.

HHV-6 primary infection in children

The primary infection of HHV-6B occurs in developed countries before three years in over 90% of the population (De Bolle *et al.*, 2005a). Only a minority of the infants has typical ES symptoms. ES symptoms in-

clude a high fever for approximately three days and a typical maculopapular rash that follows fever and resolves within three days. ES is often misdiagnosed as measles or rubella in countries where measles and rubella exist (Black *et al.*, 1996; Tait *et al.*, 1996; Oliveira *et al.*, 2003). In Finland, after the start of nationwide measles, mumps and rubella (MMR) vaccination program in 1982, pediatric encephalitides associated with these viruses had vanished by 1989 (Koskiniemi and Vaheri, 1989) and measles and rubella were eliminated in 1996 (Peltola *et al.*, 1994; Peltola *et al.*, 2000). Anyhow, most HHV-6B primary infections are asymptomatic. In some cases HHV-6B primary infection is associated with CNS symptoms, such as febrile seizures and convulsions (Caserta *et al.*, 1994; Hall *et al.*, 1994; Ward *et al.*, 2005), and even encephalopathy (Asano *et al.*, 1992; Ward *et al.*, 2005) or meningoencephalitis (Ishiguro *et al.*, 1990; Yoshikawa *et al.*, 1992; Yanagihara *et al.*, 1995). HHV-6B remains latent in blood cells and in salivary glands (Sada *et al.*, 1996; Pereira *et al.*, 2004; Chen and Hudnall, 2006). It is not known at what age the variant A is acquired.

HHV-6 primary infection in adults and reactivation/ reinfection

Primary infection, at least in the case of HHV-6B, in older children and adults is rare. Primary infection at later ages, in the light of other herpesviruses, might be expected to cause a more severe disease (Ward, 2005). Mononucleosis-like syndrome in patients negative for EBV and CMV has been suggested to be caused by HHV-6 (Steeper *et al.*, 1990; Akashi *et al.*, 1993; Goedhard *et al.*, 1995), although it is arguable whether these infections were primary infections or reactivations (Morris, 1993). Prolonged lymphadenopathy (Niederman *et al.*, 1988; Irving and Cunningham, 1990), fulminant hepatitis (Irving and Cunningham, 1990; Sobue *et al.*, 1991) and encephalomyelitis (Portolani *et al.*, 2006) have also been associated with primary HHV-6 infection in adults. HHV-6 DNA has been found in patients with sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease) (Levine *et al.*, 1992) and histiocytic necrotizing lymphadenitis (Kikuchi's disease) (Sumiyoshi *et al.*, 1993).

HHV-6 and central nervous system disease

Although usually benign, HHV-6 primary infection can invade the CNS and cause brain and spinal cord diseases (Kondo *et al.*, 1993; Caserta *et al.*, 1994; Lyall, 1996; Yoshikawa and Asano, 2000; Mannonen *et al.*, 2007). Furthermore HHV-6 reactivations and primary infections in immunocompetent elderly subjects can cause, albeit rarely, severe CNS complications such as encephalitis and myelitis (Novoa *et al.*, 1997; Portolani *et al.*, 2001; Portolani *et al.*, 2005; Portolani *et al.*, 2006). It is worth nothing that HHV-6 is frequently detected from healthy brain tissue as well (Challoner *et al.*, 1995; Chan et al., 2001; Hemling et al., 2003). The CNS complications of HHV-6 infection, febrile seizures and encephalitis, are discussed below.

HHV-6 and febrile seizures

Febrile seizures are the most common cause of seizures in early childhood and more than 80% of the patients with febrile seizures are less than three years of age (Nelson and Ellenberg, 1978). ES was linked to febrile seizures long before HHV-6 was found (Möller, 1956). After the discovery of HHV-6 and development in virological methods the association of HHV-6 and febrile seizures has strengthened, although the association is not indisputable (Zerr *et al.*, 2005b). The incidence of febrile seizures in primary HHV-6 infection has been reported to be 8% in Japanese (Asano *et al.*, 1994) and 13% in U.S. populations (Hall *et al.*, 1994).

HHV-6 encephalitis

The brain is an important site for both active and latent infection of HHV-6. Indeed, severe diseases of the brain have been reported, both during primary infection (Asano *et al.*, 1992; Kamei *et al.*, 1997; Ahtiluoto *et al.*, 2000; Rantala *et al.*, 2000; Kato *et al.*, 2003; Mannonen *et al.*, 2007) and during reactivation (Portolani *et al.*, 2002; Isaacson *et al.*, 2005; Holden and Vas, 2007). Most of the cases are reported in immunocompromised patients, but cases are reported in immunocompetent subjects as well (Novoa *et al.*, 1997; Portolani *et al.*, 2002; Isaacson *et al.*, 2005; Sawada *et al.*, 2007). Encephalitis or encephalopathy is the most common clinical manifestation of HHV-6

infection after stem cell or bone marrow transplantation (Ljungman and Singh 2006). In solid organ transplantation HHV-6 infections are not as frequent as in stem cell or bone marrow transplantation; however, encephalitis or encephalopathy is the second most common manifestation after fever and/or rash (Ljungman and Singh 2006). In some cases, HHV-6 encephalitis has been successfully treated with antivirals (Mookerjee and Vogelsang, 1997; Rieux *et al.*, 1998; Johnston *et al.*, 1999; Paterson *et al.*, 1999; Yoshida *et al.*, 2002).

HHV-6 and multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease of the CNS. The exact etiology has remained unknown but probably the disease develops as a result of the interplay between genetic and environmental factors. Several lines of evidence have suggested that infections, most probably virus infections, might act as environmental factors. Many viruses and other microbial agents have been suspected but all have failed to stand the test of time. Probably the most promising candidate is HHV-6. Preliminary reports in the early 1990s suggested possible involvement of HHV-6 in the pathogenesis of MS. Sola and co-workers (1993) investigated serum antibody titers by IFA and viral DNA in PBMCs by PCR from 126 MS patients and from 500 controls. MS patients had significantly higher serum antibody titers than controls did. Anyhow, HHV-6 DNA was found only rarely from MS patients or controls and they concluded that high serum HHV-6 antibody titers might be a consequence of immune impairment rather than HHV-6 reactivation of a latent HHV-6 infection. On the other hand, it is known that HHV-6 DNA can be detected only within a narrow time window after active infection, and thus might in part reduce the amount of positive DNA findings in this study. In another study (Wilborn *et al.*, 1994) HHV-6 DNA was detected in three cerebrospinal fluid (CSF) samples of 21 MS patients (14.3%), but not in patients with other neurological diseases (OND) including myalgic encephalitis, meningitis and chronic fatigue syndrome or in controls. HHV-6 serum antibody titers investigated by ELISA were also higher in sera of patients with MS compared to OND or controls, supporting possible involvement of HHV-6 in the pathogenesis of MS.

For the first time direct evidence to the involvement of HHV-6 in the pathogenesis of MS was reported in 1995 (Challoner *et al.*, 1995). Challoner and co-workers used representational difference analysis (RDA), introduced by Lisitsyn *et al.* (1993), a method that can be used to identify nucleic acid sequences that are unique to or present in greater numbers in diseased compared to healthy tissue. The DNA content from MS brain tissue was compared to DNA from peripheral blood leukocytes of healthy donors. By RDA they were able to identify a DNA sequence of 341 bp in size from one out of five patients that was essentially identical to the HHV-6 gene encoding HHV-6 major DNA protein. Secondly, they studied the presence of HHV-6 DNA in brain samples by nested PCR and found that HHV-6 DNA was present in 78% and 74% of MS cases and controls, respectively. Although the authors of the paper concluded that HHV-6, especially variant B is a commensal virus in the human brain, they also demonstrated HHV-6B antigen expression in MS plaques in oligodendrocytes, but neither in control brains nor in the regions other than plaques in diseased brains. Since the destruction of oligodendrocytes (leading to degradation of myelin) is a hallmark of MS, the studies suggested an association of HHV-6 with the etiology or pathogenesis of MS.

In another study (Cermelli *et al.*, 2003), MS plaques were isolated by laser microdissection from brain samples and DNA was purified and used for detection of HHV-6 DNA by nested PCR. Controls included brain samples from normal appearing white matter (NAWM) from the same patients with MS and brain samples from patients with other neurological diseases and patients without known neurological disorders. The rate of HHV-6 DNA was similar in NAWM samples and in control patient samples, but was significantly higher in MS plaques. Others have studied HHV-6 antigen expression in MS brain samples as well. Carrigan and Knox with others (Carrigan and Knox, 1997; Knox *et al.*, 2000) found HHV-6 antigen in eight out of 11 brain samples from patients with MS but not in any of the seven control brain samples. The result was confirmed by Friedman *et al.* (1999), but Coates and Bell (1998) were not able to identify HHV-6 antigen in any of the 23 brain samples from patients with MS; albeit the successfull

identification of HHV-6 antigen in salivary gland tissue.

Many other infectious agents have been associated with MS. Many of them have not stood the test of time. Two other herpesviruses VZV (Sotelo et al., 2008) and EBV have been also associated with MS. Nearly 100% of patients with MS are seropositive for EBV compared with 90-95% in adults (Sundstrom *et al.*, 2004; Levin *et al.*, 2005). Furthermore, individuals with a history of symptomatic EBV infection have a two-fold higher risk to develop MS compared to individuals with asymptomatic infection (Thacker *et al.*, 2006; Nielsen *et al.*, 2007). In addition, human endogenous retrovirus (HERV), MS-associated retrovirus, has been associated with the disease (Mameli *et al.,* 2007).

Increased antibody titers in serum to HHV-6 reported by Sola *et al*. (1993) have been also confirmed by other investigators (Wilborn *et al.*, 1994). Anyhow, antibody findings in both serum and CSF summarized in Table 2 are controversial.

Table 2. Detection of HHV-6 antibodies in patients with MS compared to control patients

Sample	Reference	MS (%)	Control (%)
Serum IgG	(Sola *et al.*, 1993)	71	41
	(Wilborn *et al.*, 1994)	high titers	low titers
	(Liedtke *et al.*, 1995)	39	18
	(Nielsen *et al.*, 1997)	equal titers	equal titers
	(Soldan *et al.*, 1997)	85	72
	(Ablashi *et al.*, 1998)	69	28
	(Enbom *et al.*, 1999)	100	100
	(Ongradi *et al.*, 1999)	higher titers	lower titers
	(Ablashi *et al.*, 2000)	90	75
	(Taus *et al.*, 2000)	30	25
	(Xu *et al.*, 2002)	equal titers	equal titers
	(Derfuss *et al.*, 2005)	84	88
	II	100, higher titers (6A) 30, equal titers (6B)	69, lower titers (6A) 38, equal titers (6B)
	(Kuusisto *et al.*, 2008)	88	86
Serum IgM	(Liedtke *et al.*, 1995)	3	2
	(Soldan *et al.*, 1997)	73	18
	(Ablashi *et al.*, 1998)	56	19
	(Friedman *et al.*, 1999)	80	16
	(Ongradi *et al.*, 1999)	higher titers	lower titers
	(Ablashi *et al.*, 2000)	71	15
	(Enbom *et al.*, 2000)	2	NT
	(Taus *et al.*, 2000)	0	0
	(Xu *et al.*, 2002)	equal titers	equal titers
	(Kuusisto *et al.*, 2008)	6	0
CSF IgG	(Sola *et al.*, 1993)	7	NT
	(Wilborn *et al.*, 1994)	0	0
	(Ablashi *et al.*, 1998)	39	7
	(Friedman *et al.*, 1999)	94	100
	(Ongradi *et al.*, 1999)	43 (6A) 87 (6B)	17 (6A) 0 (6B)
	(Ablashi *et al.*, 2000)	4	NT
	(Derfuss *et al.*, 2005)	34	12
	II	15 (6A) 0 (6B)	0 (6A) 0 (6B)
	(Kuusisto *et al.*, 2008)	0	0
CSF IgM	(Friedman *et al.*, 1999)	0	0
	(Ongradi *et al.*, 1999)	0 (6A) 57 (6B)	0 (6A) 0 (6B)
	(Ablashi *et al.*, 2000)	0	0
	(Kuusisto *et al.*, 2008)	0	0

HHV-6 DNA findings in different sample materials from patients with MS compared to different controls vary between studies as well (Table 3). DNA detection rates vary from 0 to 83% and 0 to 53% in serum in cases and controls, respectively. In CSF the detection rates are from 0 to 78% and 0 to 20% in cases and controls, respectively. Only a few studies have reached a statistically significant difference between groups. In brain samples the detection rates are higher than in serum or CSF, although only a slight increase in positive cases are seen in MS compared to controls.

Still, to date, only few studies have evaluated the possible mechanisms by which HHV-6 might be involved in the pathogenesis of MS, albeit many reviews exist (Clark, 2004). It has been postulated that HHV-6 might induce a phenomenon known as molecular mimicry (Fotheringham and Jacobson, 2005). The residues 4-10 from HHV-6 U24 gene-encoded hypothetical protein are identical to myelin basic protein residues 96-102 (Tejada-Simon *et al.*, 2003). In addition, the synthetic peptide corresponding residues 1-13 of U24 was able to induce activation in more than 50% of the T-cells recognizing myelin basic protein residues 93-105. The frequency of the subpopulation of T-cells recognizing both peptides was significantly increased in patients with MS compared to healthy controls.

Table 3. Detection of HHV-6 DNA in patients with MS compared to control patients

Sample	Reference	MS (%)	Control (%)
Serum DNA	(Wilborn *et al.*, 1994)	0	0
	(Martin *et al.*, 1997)	0	NT
	(Soldan *et al.*, 1997)	30	0
	(Fillet *et al.*, 1998)	6	0
	(Goldberg *et al.*, 1999)	4	0
	(Mirandola *et al.*, 1999)	0	0
	(Tejada-Simon *et al.*, 2002)	67	33
	(Al-Shammari *et al.*, 2003)	0	0
	(Tejada-Simon *et al.*, 2003)	83	55
	II	0	0
	(Kuusisto *et al.*, 2008)	0	0
PBMC DNA	(Sola *et al.*, 1993)	3	4
	(Torelli *et al.*, 1995)	3	22
	(Merelli *et al.*, 1997)	5	0
	(Mayne *et al.*, 1998)	25	24
	(Rotola *et al.*, 1999)	41	29
	(Ablashi *et al.*, 2000)	75	60
	(Hay and Tenser, 2000)	7	14
	(Kim *et al.*, 2000)	21	0
	(Rotola *et al.*, 2000)	40	37
	(Taus *et al.*, 2000)	14	0
	(Chapenko *et al.*, 2003)	62	29
Plasma DNA	(Chapenko *et al.*, 2003)	31	0
CSF DNA	(Wilborn *et al.*, 1994)	14	0
	(Liedtke *et al.*, 1995)	11	5
	(Martin *et al.*, 1997)	0	0
	(Ablashi *et al.*, 1998)	17	0
	(Fillet *et al.*, 1998)	6	0
	(Enbom *et al.*, 1999)	6	6
	(Goldberg *et al.*, 1999)	0	0
	(Mirandola *et al.*, 1999)	0	0
	(Taus *et al.*, 2000)	0	0
	(Tejada-Simon *et al.*, 2002)	47	20
	(Cirone *et al.*, 2002)	78	NT
	II	0	0
	(Kuusisto *et al.*, 2008)	0	0
Brain DNA	(Challoner *et al.*, 1995)	78	74
	(Sanders *et al.*, 1996)	57	38
	(Merelli *et al.*, 1997)	0	50
	(Coates and Bell, 1998)	equal levels	equal levels
	(Friedman *et al.*, 1999)	36	14
	Hemgling et al., (2003)	NT	88
	(Cermelli *et al.*, 2003)	58	27

HHV-6 and epilepsy

CNS infections have been associated with epilepsy. Dr. Uesugi with others (2000) was the first to suggest that HHV-6 might be a cause of temporal lobe epilepsy. They found that 6 out of 16 (35%) temporal lobe resections were positive for HHV-6. In another study, HHV-6 was found in 4 out of 8 mesial temporal lobe resections, but not in any of the 7 neocortical epilepsy brain samples (Donati *et al.*, 2003). HHV-6 was identified to be variant B in all of the cases and infected cells were shown by immunohistochemistry to be GFAP-positive astrocytes. In subsequent studies the investigators studied 16 patients more and found that 11 of these (69%) were positive for HHV-6B (Fotheringham *et al.*, 2007). They were able to isolate primary astrocytes and culture them *in vitro*, and found that the astrocytes expressed HHV-6 antigen and were HHV-6B DNA positive. Furthermore, they infected astrocyte cultures with HHV-6B and showed that infection resulted as glutamate transporter dysfunction providing a possible mechanism for the induction of mesial temporal lobe epilepsy symptoms (Fotheringham *et al.*, 2008).

HHV-6 in immunocompromised patients

HHV-6 in AIDS

HHV-6, especially HHV-6A, has been suggested to play a part in AIDS as a cofactor or a progression factor (Lusso and Gallo, 1994; Lusso and Gallo, 1995). HHV-6 and HIV-1 have a tropism for $CD4^+$ T cells (Lusso *et al.*, 1988; Lusso *et al.*, 1989) and HHV-6 might partially contribute to the loss of these cells. Furthermore, HHV-6 is capable of transactivating the HIV-1 LTR promoter thus stimulating HIV-1 infection (Ensoli *et al.*, 1989; McCarthy *et al.*, 1998). HHV-6 might indirectly enhance HIV infection by promoting secretion of cytokines that are known to enhance HIV infection, *i.e.* interleukin-1β and tumor necrosis factor-α (Flamand *et al.*, 1991). HIV uses the receptor complex formed by CD4 and chemokine receptor CCR5 or CXCR4. Interestingly, HHV-6 infection in lymphoid tissues *ex vivo* suppresses CCR5-tropic HIV-1 infection, but not CXCR4-tropic HIV-1 (Grivel *et al.*, 2001). This might lead to HIV infection through CXCR4 coreceptor which is linked to more aggressive progression of HIV infection to AIDS. In ma-

caques (*Macaca nemestrina*) co-infection by HHV-6A and simian immunodeficiency virus (SIV) results in accelerated progression of AIDS accompanied with depletion of both $CD4^+$ and $CD8^+$ T cells (Lusso *et al.*, 2007).

HHV-6 in transplantation

HHV-6 infection is frequently detected in immunosuppressed patients and in most cases the infection is HHV-6B reactivation (Drobyski *et al.*, 1993; Wang *et al.*, 1999). The incidence peaks at two to three weeks after transplantation (Singh and Carrigan, 1996). The incidence of HHV-6 infection varies greatly between studies and is 28 to 75% for bone marrow transplant recipients and 0 to 85% for solid organ transplant recipients (De Bolle *et al.*, 2005a). HHV-6 induced encephalitis and encephalopathy are the most common clinical diseases after stem cell or bone marrow transplantation (Drobyski *et al.*, 1994; Wang *et al.*, 1999; Yoshida *et al.*, 2002) following pneumonitis (Carrigan *et al.*, 1991; Cone *et al.*, 1993) and delayed engraftment (Johnston *et al.*, 1999). There are reports on rash development (Yoshikawa *et al.*, 1991), bone marrow suppression (Carrigan and Knox, 1994; Hentrich *et al.*, 2005), and graft versus host disease (GVHD) (Appleton *et al.*, 1995; Hentrich *et al.*, 2005; Zerr *et al.*, 2005a). In liver transplantation, clinical manifestations of HHV-6 infection include fever and/or rash, encephalitis or encephalopathy (Sutherland *et al.*, 1991; Montejo *et al.*, 2002), hepatitis (Ward *et al.*, 1989; Humar *et al.*, 2002), graft dysfunction or rejection (Okuno *et al.*, 1990; Lautenschlager *et al.*, 1998; Griffiths *et al.*, 1999; Lautenschlager *et al.*, 2000), and bone marrow suppression or pneumonitis (Singh *et al.*, 1997). In addition, HHV-6B can infect gastroduodenal mucosa after liver transplantation (Halme *et al.*, 2008). HHV-6B has been shown to infect kidney transplants as well (Helanterä *et al.*, 2008).

HHV-6 transmission

HHV-6B is probably in most cases transmitted horizontally via saliva from parents or siblings to the child (Mukai *et al.*, 1994; Di Luca *et al.*, 1995; van Loon *et al.*, 1995; Tanaka-Taya *et al.*, 1996; Pereira *et al.*, 2004). Virtually all of the isolates from the saliva have been variant B (Collot *et al.*, 2002)

which might suggest that HHV-6A is not transmitted through saliva. In addition, vertical transmission by congenital infection occurs in approximately 1-2% of the cases. Congenital infection may occur either by virus crossing the placenta or may be hereditary if virus is integrated into human chromosomes (Ward, 2005). As mentioned earlier in the Structure and genome section HHV-6 contains an array of sequences similar to those present in telomers of mammalian chromosomes (Gompels and Macaulay, 1995; Gompels *et al.*, 1995). Although the exact role of these telomeric repeats at the DRs is not known, it has been suggested that they might be involved in site-specific integration of the HHV-6 genome to the site near chromosomal telomers in mammalian genome (Torelli *et al.*, 1995; Daibata *et al.*, 1998). Interestingly, HHV-6 encodes a protein that has significant homology to adeno-associated virus (AAV) rep protein (Thomson *et al.*, 1991) which has a role in the integration of the AAV to the host genome (Linden *et al.*, 1996). Thus it is assumed that HHV-6 AAV rep homology protein might possess similar functions in the integration of HHV-6 into the host genome (Ward, 2005).

Congenital infection detected as DNA in cord blood cells has been reported in 1.6% of German births (Adams *et al.*, 1998) and 0.9% of Swedish births (Dahl *et al.*, 1999). Interestingly, it has been published that in 0.2% of Japanese births HHV-6 was transmitted from either of the parents to the child (Tanaka-Taya *et al.*, 2004). In another study, Ward and others (2007) identified chromosomally integrated HHV-6 in 1.3% of older children and adults. The percentage of adults having HHV-6 genome integrated into their genomes is approximately in the same order as the percentage of congenital infections suggesting that most congenital infections might occur by inheritance rather than virus crossing through placenta. Although one report describes severe neurological symptoms related to intrauterine transmission (Lanari *et al.*, 2003), most of the intrauterine transmissions does not cause significant risk (Boutolleau *et al.*, 2003; Hall *et al.*, 2004). HHV-6 DNA has been found in genital tract of approximately 20% or less of pregnant women (Okuno *et al.*, 1995; Maeda *et al.*, 1997; Ohashi *et al.*, 2001; Caserta *et al.*, 2007), but perinatal transmission seems unlike-

ly. The common transmission route among young children, fecal-oral, has not been documented in the case of HHV-6, although HHV-6 has been reported to be excreted into stool persistently or intermittently (Suga *et al.*, 1995; Suga *et al.*, 1998). Despite the fact that virtually all postnatal HHV-6 infections in young children are caused by HHV-6B (Dewhurst *et al.*, 1993a) as much as one third of all congenital infections are caused by HHV-6A (Hall *et al.*, 2004).

Antiviral susceptibility

Unfortunately, to date no controlled clinical trials of antiviral activity against HHV-6 have been carried out (Clark and Griffiths, 2003; De Bolle *et al.*, 2005a) and no drugs have been formally approved for the treatment of HHV-6 infections. In general, antiviral compounds effective against another betaherpesvirus, CMV, show similar potency against HHV-6 *in vitro*. Therefore, the drugs clinically used are the same that are used for the treatment of HCMV infections. The drugs that are potentially effective against HHV-6, namely ganciclovir, foscarnet and cidofovir, all have their side effects and should not be, unless in life-threatening situations, used for the treatment of immunocompetent patients.

In vitro, ganciclovir, a guanine analogue, has superior activity against HHV-6 compared to acyclovir or penciclovir that is independent of the propagation system used (Burns and Sandford, 1990; Agut *et al.*, 1991; Yoshida *et al.*, 1998; Manichanh *et al.*, 2000). The pyrophosphate analogue foscarnet, and phosphonate cidofovir have both been shown to have antiviral activity against HHV-6 (Agut *et al.*, 1991; Reymen *et al.*, 1995). HHV-6 lacks a viral thymidine kinase and therefore thymidine kinase-dependent drugs such as acyclovir have little or no effect against HHV-6 *in vitro* (Agut *et al.*, 1991; Reymen *et al.*, 1995).

In vivo several case reports have showed that ganciclovir is effective against HHV-6 encephalitis in patients who underwent bone marrow or solid organ transplantation (Mookerjee and Vogelsang, 1997; Rieux *et al.*, 1998; Johnston *et al.*, 1999; Paterson *et al.*, 1999; Yoshida *et al.*, 2002). In few cases the treatment with ganciclovir has shown no response (Tiacci *et al.*, 2000; Rossi *et*

al., 2001). Interestingly, the case presented by Rossi *et al.* (2001) appeared to be a primary HHV-6A infection of an adult woman and this might have affected the outcome. Although lacking *in vitro* antiviral activity, acyclovir in high doses reduced the proportion of HHV-6 DNA-positive blood samples in bone marrow transplant patients and tended to lower the HHV-6 DNA levels compared to those who were not treated with the drug (Wang *et al.*, 1996).

Viral neurological infections

Viral infections of the CNS occur worldwide and are most prevalent in young ages (Koskiniemi *et al.*, 1991; Koskiniemi *et al.*, 2001). Inclusion of conventional childhood viruses, such as measles, mumps and rubella into the vaccination program in Finland in 1982 has changed the spectrum of the causative agents of viral encephalitis. The emergence of new viruses or new virus strains will change our view even more in the future, not to mention development of more sensitive molecular diagnostic methodology. VZV and enteroviruses seem to be the most common causes of various CNS infections following herpes simplex viruses in Finland (Figure 5) (Koskiniemi *et al.*, 2001; Kupila et al., 2006). Enteroviruses are the most common cause of aseptic meningitis (Kupila et al., 2006). Encephalitis, meningitis, myelitis, vasculopathy, ganglioneuritis, retinal necrosis and optic neuritis can be caused by one or more members of the HHV family (Gilden *et al.*, 2007) and jointly the family comprises more than half of identified pathogens in viral CNS infections (Koskiniemi *et al.*, 2001).

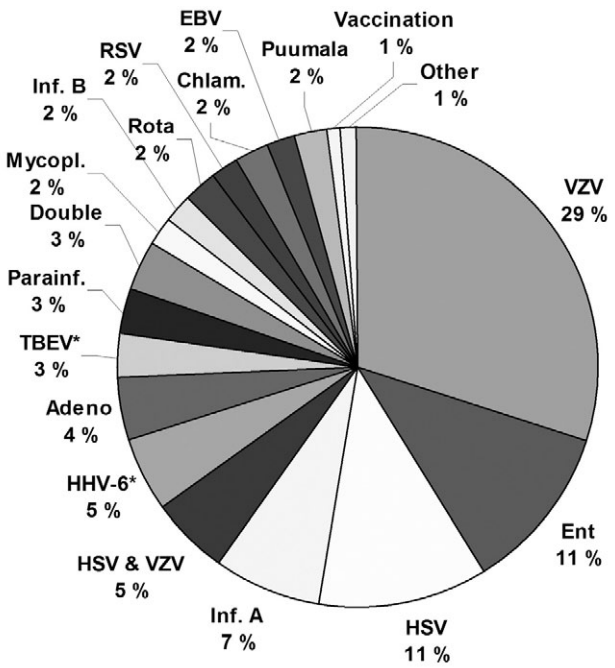

*Figure 5. Proportions of findings (confirmed or probable) to different viruses among patients with CNS infection in Finland. *Only selected samples were studied. (Koskiniemi et al., 2001)*

Multiple sclerosis

Multiple sclerosis (MS) is the most common neurological cause of disability in young adults.

Pathogenesis

MS is a chronic demyelinating disease of the CNS with unknown etiology. The disease is most probably caused by interplay between genes and environmental factor(s). Certain genes probably increase the risk of developing MS, but environmental factors trigger the development and cause relapses, typical of MS. Anyhow, epidemiological and immigration studies have suggested that not only genetic factors increase the risk, but also environmental factors, including viruses might increase risk to develop the disease (Kurtzke, 1993).

The mechanisms of demyelination, the primary feature of MS, are heterogeneous and vary between patients. The axons are relatively conserved during the disease, but the axon potentials, electrical communication system between neurons, are transmitted insuf-

ficiently, because of lack of myelin (Lassmann *et al.*, 2001). Myelin is a protein component produced by brain supporter cells, oligodendrocytes (Figure 6). In normal myelinated tracts most axons are wrapped by myelin sheaths made from layers of cell membrane. One oligodendrocyte can contribute up to 40 myelin sheaths to nearby axons (ffrench-Constant, 1994).

Proteolytic activation has been associated with demyelination process in multiple sclerosis. Matrix metalloproteinases (MMPs) and plasminogen activators have been shown to be both beneficial as well as detrimental enzymes within the CNS taking part in both physiological and pathophysiological processes (Teesalu et al., 2002; Agrawal et al., 2008). Elevated levels of tissue plasminogen activator (tPA) have been reported in MS CSF (Akenami et al., 1996). In experimental autoimmune encephalomyelitis (EAE), a widely used animal model for MS, activation of MMPs and plasminogen activators have been reported (Teesalu et al., 2001).

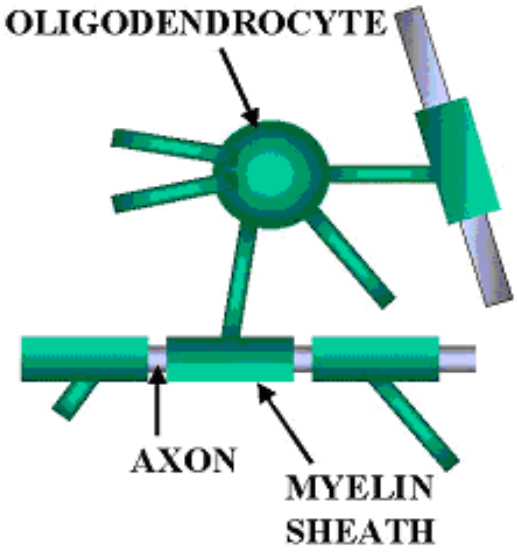

Figure 6. Oligodendrocytes produce myelin, which is composed of multiple layers of oligodendrocyte membranes that wrap concentrically around one or more axons. The myelin sheath around an axon acts like an electrical insulator, allowing nerve impulses to be conducted very quickly. Myelin appears white and shiny because the layers of membrane that form it contain large amounts of lipids (ffrench-Constant, 1994).

There are at least four different pathogenetic mechanisms of MS that can be identified from pathology of brain samples causing MS (Lassmann *et al.*, 2001). The mechanisms are presented in Figure 7. The first step in all four mechanisms is inflammation, caused by T-cells and macrophages, which can lead by four different ways to either demyelination or to programmed cell death, apoptosis, of oligodendrocytes. In the first pattern the macrophages mediate demyelination and induce the release of cytokines, such as tumor necrosis factor α (TNF-α), reactive oxygen intermediates (ROI) and proteinases. In the second pattern antibodies

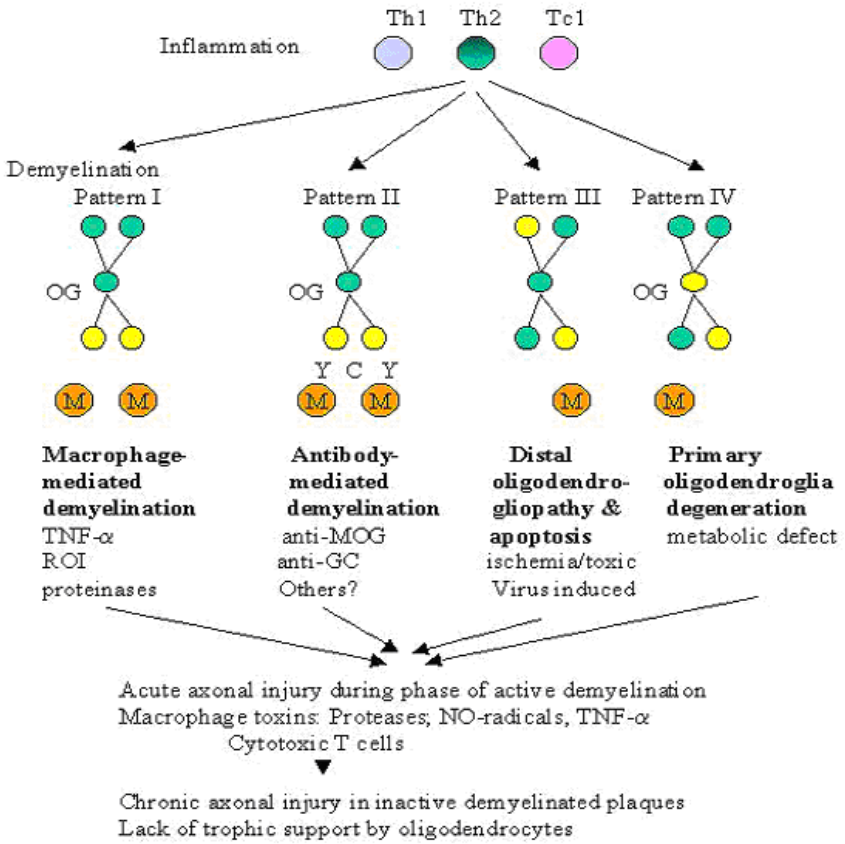

Figure 7. Different pathogenetic mechanisms involved in the formation of MS lesions. Green spheres represent normal myelin sheaths and yellow spheres demyelinated myelin sheaths produced oligodendrocytes (OG). Y, antibody, C, Complement, Th1, T-helper cell 1, Th2, T-helper cell 2, Tc1, cytotoxic T cell 1 (Lassmann et al., 2001).

raised against the myelin oligodendrocyte unit, such as anti-myelin oligodendrocyte glycoprotein (anti-MOG) and anti-galactocerebroside (anti-GC) cause demyelination. The third pattern is caused by different toxins, or ischemia, or by infection of viruses. These lead to apoptosis of oligodendrocytes. The fourth pattern is caused by deficiency in oligodendrocyte metabolic pathways as a result of macrophage toxins or a genetic defect.

B-cells and humoral immune response in multiple sclerosis

MS is predominantly considered a T-cell mediated disease due to the fact that MS plaques contain large amounts of T-cells. However, B-cells have gathered considerable impact in the pathogenesis recently. Indeed, B-cells might contribute to the pathogenesis at many levels starting from the earliest antigen capture, the processes of tissue destruction, and even continuing in remyelination and tissue repair (Duddy and Bar-Or, 2006).

One of the features of the pathogenesis of MS is the production of chronic intrathecal immunoglobulins (Ig) presenting as oligoclonal IgG bands (OCB) in cerebrospinal fluid. The clonality of the IgG in brain and CSF seems to be stable over long periods in MS (Walsh and Tourtellotte 1986). Anyhow, it has been shown that the prevalence of MS patients with positive OCBs increase over time suggesting that activated B-cells in early disease are not yet residents of the CNS (Petereit and Reske 2005). In addition, there are a number of low-affinity IgGs present in CSF towards various infectious agents, such as measles, rubella, VZV and *Chlamydia pneumoniae* (Sindic *et al.*, 1994; Luxton *et al.*, 1995; Reiber *et al.*, 1998; Derfuss *et al.*, 2001). Low-affinity IgGs present in CSF as well as in serum in similar patterns. Low-affinity IgGs are hypothesized to be part of polyclonal B-cell activation (Derfuss *et al.*, 2001). However, polyclonal B-cell activation, *per se*, is not sufficient to produce OCBs. The specificity of the OCBs is not known.

It has been suggested that if virus(es) cause MS, it should be possible to demonstrate that OCBs contain IgG bands that are directed to a given virus (Gilden, 2001). However, due to the heterogeneity of MS it is not likely to find a single virus

that causes all of the MS cases. Indeed, it has been shown that some of the patients with MS have OCBs that are directed against EBV. Rand *et al.* (2000) showed that one third of the 15 patients with MS had OCBs directed against EBNA-1 in their CSF, although critically analyzing the results only two patients had CNS-specific OCBs directed against EBV, since other three had similar bands in their serum as well. In another study two EBV proteins were shown to react with OCBs (Cepok *et al.*, 2005). In addition, measles antigen might react with OCBs as well (Vandvik et al., 1976).

Epidemiology

The geographic distribution of MS is unique, the disease being more common in temperate zones than near the equator. MS is most common in populations of Northern Europe descent, especially in Scandinavia, the British Isles, the Northern regions of the United States and Southern Canada (Compston, 1997), and it has even been postulated that Vikings might have been the ancestor in dissemination of the genetic susceptibility to the disease (Poser, 1994; Poser, 1995). Anyhow, genetic susceptibility cannot exclusively explain the uneven geographical distribution, suggesting the involvement of environmental factors in the onset of the disease. Large-scale immigrant studies have shown that the risk of MS decreases if children migrates from a high- to low-risk region and increases when children migrates from a low- to high-risk region, in contrast to rates for nonmigrants at their birthplaces (Kurtzke, 1993). In generally, this applies to children under the age of 14. Immigrants older than 15 years have already acquired the risk from the homeland. These studies suggest that environmental factors are as important factors as genetic susceptibility in the development of the disease. Finland belongs to a high-risk area of MS with a prevalence of approximately 100/100000 (Sumelahti *et al.*, 2002). MS cases seem to cluster to the Western part of Finland and approximately a two times higher prevalence compared to the national average has been reported in Seinäjoki in Southern Ostrobothnia (Sumelahti *et al.*, 2001).

Aims of the study

The aims of the present PhD studies were to investigate neurological infections possibly associated with HHV-6. This virus has been associated with MS and a major goal was to clarify the role of HHV-6 in the pathogenesis of MS. The itemized aims were as follows:

1. To study the HHV-6 antigen expression in multiple sclerosis brain tissue and compare it to brain tissue obtained from healthy controls. Furthermore, we aimed to study the expression of tPA, an enzyme implicated in the demyelination process in MS, in relation to the expression of HHV-6 antigen.

2. To study the serological evidence of the role of HHV-6 in the development of MS and measure HHV-6 antibodies in serum and CSF samples from patients with MS and compare the levels with patients with OND.

3. To study the presence of oligoclonal bands in patients with MS and serologically suspected HHV-6 infection and the specificity of OCBs in regard to HHV-6 antigen.

4. To study the relationship between HHV-6 primary infection and neurological symptoms in children with suspected encephalitis.

Materials and methods

Patients, samples and controls (I-IV)

Table 4. Samples used in studies I-IV

Study	I	II	III	IV
Brain tissue	15 (MS) 10 (controls)	-	-	-
Serum	-	27 (CDMS) 19 (CPMS) 26 (OND)	*4 (CDMS) 5 (CPMS)	53 (suspected encephalitis)
CSF	-	27 (CDMS) 19 (CPMS) 27 (OND)	4 (CDMS) 5 (CPMS)	**4 (suspected encephalitis)

*patients with positive HHV-6 IgG CSF or patients with low-avidity antibodies in serum in study II were enrolled to study III, **only CSF samples corresponding HHV-6 low-avidity IgG serum samples were studied

Samples used in studies I-IV are presented in Table 4. Brain tissue specimens from chronic plaques of the patients with MS and control brain tissue specimens were acquired through Professor John Zabriskie from Rockefeller University, New York, NY, USA. All of the brain specimens were from U.S. patients. Serum and CSF samples from patients with MS and patients with OND were collected from the Neurological Outpatient Department of Helsinki University Central Hospital during the years 2003 and 2004. Serum was separated from the whole blood and CSF samples were centrifuged at 2700 g for 10 minutes to obtain cell-free CSF. All samples were aliquoted, frozen and stored at -70 °C until used. The total number of patients was 126. In a retrospective patient analysis 27 appeared to have a clinically definite MS (CDMS), diagnosed using the McDonald criteria (McDonald *et al.*, 2001) revised by Polman *et al.* (2005). 19 patients had clinically possible MS (CPMS). Reference patients group was formed from patients with various other neurological diseases and symptoms (OND) with matching gender and ages (± 5 yrs) to each patient with CDMS. A total of 46 patients with MS (either CDMS or CPMS) and reference group of 27 patients with OND were included in the

studies. One serum sample from patient with OND was not available. Otherwise serum and CSF samples were available from all of the patients with CDMS, CPMS, and OND. All patients gave a written consent and the Ethics Committee of Helsinki University and Helsinki University Central Hospital approved the study.

Sera of 53 children less than 15 years old were collected from a panel sent to the Department of Virology, University of Helsinki, for suspected viral encephalitis. The prospective study covered more than two thirds of the whole country and lasted for a two-year period 1995–1996 (Koskiniemi *et al.*, 2001). The samples for the study of HHV-6 were consecutively collected from the first and last quarter of the year 1995.

Immunohistochemistry and in situ hybridization (I)

Immunohistochemical staining and *in situ* hybridization were carried out using automatic slide stainers Ventana Gen II™ and Ventana Discovery™ (Ventana Medical Systems Inc., Tucson, AZ, USA). Brain tissue specimens from MS plaques of 15 patients and from respective areas of ten controls were fixed in formalin and embedded in paraffin. Serial 6-µm sections were cut from the paraffin blocks for immunohistochemistry and 3-4-µm thick sections for *in situ* hybridization. Viral antibodies used were: mouse monoclonal to HHV-6B virion protein 101 kDa (Pellett *et al.*, 1993) (Chemicon International Inc, Temecula, CA, USA), HHV-6A gp200 (Chemicon) and CMV clone CCH2 (DAKO, Carpinteria, CA, USA). Cell markers used were rabbit anti glial fibrillary acidic protein (GFAP) (DAKO) for astrocytes and rabbit anti myelin basic protein (MBP) (DAKO) for oligodendrocytes.

Epstein-Barr in situ hybridization was done using an FITC-conjugated EBER PNA probe (DAKO).

Immunofluorescence assays (II, IV)

HHV-6A strain GS was grown in HSB-2 T-cell line and HHV-6B strain Z-29 was grown in MOLT-3 T-cell line. Equal amount of infected cell supernatant from previous infection and mixture of uninfected cells were mixed together. The final cell concentration was approximately 10^6 cells/ml. After the

$$avidity = \frac{w/oUREA}{wUREA} \text{ ,low-avidity } > 8 \quad (1)$$

$$avidity\% = \frac{wUREA}{w/oUREA} \cdot 100\% \text{ ,low-avidity } < 12.5\% \quad (2)$$

Equation (1) was used in study **II** *and equation (2) was used in study* **IV**.

infection fetal bovine serum concentration was lowered to 2% or to 0.5%, in HHV-6A and HHV-6B infections, respectively, and 5 µg/ml hydrocortisone was added. After six to ten days post infection cells were mixed with uninfected cells to achieve a proportion of infected cells of approximately 30 per cent. Approximately 6 x 10^4 cells per well were spotted on a 10-well (8-mm diameter) diagnostic glass slides and air-dried. After fixation with acetone the slides were stored at -20°C until used. Panels of known positive and negative sera were used to validate each patch of slides prepared.

Serum samples were diluted either two- or four-fold dilutions in PBS and applied to wells. Slides were incubated 30 min at 37°C and washed three times. Bound antibodies were detected with FITC-conjugated anti-human IgG secondary antibody (Monosan, Uden, The Netherlands) and counterstained with Evan's Blue.

Avidity measurements (II, IV)

Avidity was determined using IFA method essentially as mentioned above with one additional step in which the bound antibodies from the serum were washed with 8 M urea to remove low-affinity antibodies. The end-point titer was compared to one without urea wash and the avidity was calculated using the following equations:

Measurement of intrathecal antibody production (II)

Serum HHV-6 IgG antibody titers were compared to the corresponding CSF HHV-6 IgG antibody titers. Ratio below 20 suggests ITAP (Levine *et al.*, 1978):

$$\frac{serumAb}{CSFAb} \text{ ,marker of ITAP } < 20 \quad (3)$$

The antibody index (AbI) was used to determine CNS-based production of HHV-6 specific antibodies. The antibody ratio

between serum and CSF was corrected against total IgG amount in serum and CSF using the following equation:

$$AbI = \frac{CSFAb / SAb}{CSFIgG / SIgG}, > 1.5 \text{ pathological finding} \quad (4)$$

Isoelectric focusing (IEF) and immunofixation (III)

Paired serum/CSF samples were diluted to the same IgG concentration (20 mg/L IgG) and 10 µl of sample was applied to isoelectric focusing. Samples were isoelectrically focused using Sebia Hydragel 3 CSF Isofocusing system on the Sebia Hydrasys Focusing apparatus (Sebia, Lisses, France). IgG bands were localized by immunofixation with peroxidase conjugated anti-IgG antiserum (Sebia) and visualized using immunoperoxidase staining using reagents from Sebia.

Affinity-driven immunoblot (III)

Nitrocellulose membrane was coated with HHV-6A (Advanced Biotechnologies, Columbia, MD, USA), HHV-6B (Meridian Life Science, Saco, ME, USA) or sucrose gradient ultracentrifugation purified HSV-1 viral antigen for 30 min and non-specific binding sites were blocked with milk casein. The membrane was overlaid with isoelectrically-focused serum/CSF samples in a thin agarose gel. Passive transfer with one kilogram weight was allowed to proceed for 30 min. Transferred bands were detected using alkaline-phosphatase conjugated anti human IgG (DAKO) and visualized using NBT/BCIP substrate solution. HSV-1 and mumps viral antigens as well as casein-blocked membranes were used as controls to ensure the specificity of the transferred IgG bands.

DNA microarray (II)

DNA was extracted from 200 µl of serum or CSF samples using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Basel, Switzerland). Multiplex-PCR and microarray was carried out as published (Jääskeläinen *et al.*, 2006; Jääskeläinen *et al.*, 2008). Briefly, two multiplex-PCR reactions were used; one to identify HSV-1 and HSV-2 and one to identify CMV, EBV, VZV, HHV-6A, HHV-6B and HHV-7 viral sequences. The multiplex-PCR products were pooled before transcribing into ssRNA using AmpliScribe™ T3-Flash™ Transcription Kit (Epicentre

Biotechnologies, Madison, WI, USA). The ssRNA samples were hybridized with specific oligonucleotide probes for each herpesvirus for 20 min at 42°C and microarray slides were washed. The primer extension was carried out with CY5-labeled nucleotides 20 min at 52°C. After washing the microarrays were analyzed using a ScanArray Express scanner, and ScanArray™ and QuantArray™ software (Perkin-Elmer, Wellesley, MA, USA). Commercial viral DNA samples were used as controls in each microarray experiment.

DNA extraction and variant-specific PCR (IV)

DNA was extracted from 200 µL of CSF and serum samples, and eluted in 80 µL of DNase-treated water. DNA was extracted using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Positive controls comprised of HHV-6A (strain U1102) and 6B (strain Z29) supernatant. Primary and nested PCR for Major Capsid Protein (MCP) and Large Tegument Protein (LTP) were conducted as previously described (Secchiero *et al.*, 1995; Akhyani *et al.*, 2000). Viral load was determined by quantitative real-time PCR as previously described (Nitsche *et al.*, 2001).

Results and discussion

Immunohistochemical and in situ hybridization analysis of MS brain samples (I)

HHV-6 variant B antigen expression was identified in 67% (10/15) brain samples from patients with MS. From ten control brain samples, three expressed HHV-6 antigen as well ($p = 0.07$). All of the samples were negative for CMV. Our results were in line with other reports (Challoner *et al.*, 1995; Friedman *et al.*, 1999). HHV-6 expression was identified mainly in oligodendrocytes, but also in astrocytes, macrophages and microglial cells, identified by cell markers using double staining techniques. It seems that, although HHV-6 is a commensal in CNS, it is more prevalent in plaques that are involved in the demyelination process. Basically, the detection of HHV-6 antigen in brain sections suggests that HHV-6 is in an active replicative form, not in a latent form. This might indicate that actively replicating HHV-6 is involved in the plague formation in MS brains.

Based on earlier observations that plasminogen activation is associated with demyelination process, we studied the expression of tPA antigen. tPA staining of the brain samples revealed that 7 out of 15 MS brain samples expressed strongly tPA, but in all control samples tPA expression was only weak or moderate and restricted to blood vessel walls ($p = 0.01$). Furthermore, double staining with HHV-6 and tPA showed that of seven samples with high tPA expression five expressed HHV-6 antigen as well. In conclusion, five out of 15 brain samples from patients with MS concomitantly expressed HHV-6 antigen and tPA compared to none of the brain samples from the control patients ($p = 0.04$). It is possible that active HHV-6 infection induces the expression of tPA in CNS. Another possibility is that tPA is an epiphenomenon related to inflammation normally observed within plaques. tPA is an important enzyme in neurogenesis and neuronal remodeling as well as in neuronal degeneration (Teesalu *et al.*, 2002; Teesalu *et al.*, 2004). It affects the CNS in two ways: it activates a zymogen, plasminogen, to active protease plasmin and tPA have also direct

effects in CNS. It have been suggested that tPA in moderate levels is physiologically beneficial to CNS, but exaggerated levels of tPA could lead to tissue damage (Teesalu *et al.*, 2002). In addition, tPA have been associated with neurological diseases such as MS showed by elevated levels of tPA in CSF (Akenami *et al.*, 1996)

All of the RNA-intact samples, shown by in situ hybridization using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (positive control probe, DAKO), were negative for EBER RNA. From five samples from patients with MS and from three samples from control patients RNA appeared to be degraded.

HHV-6 antibodies and DNA in serum and CSF of patients with MS (II)

Eighteen of 26 (69.2%) serum samples from patients with OND contained HHV-6A antibodies. All 27 patients with CDMS ($p = 0.001$) and all 19 patients with CPMS ($p = 0.007$) had HHV-6A antibodies in serum. No significant differences in HHV-6B prevalences in different patient groups were found; ten out of 27 (37.0%) patients with CDMS, four out of 19 (21.1%) patients with CPMS and ten out of 26 (38.5%) patients with OND had HHV-6B antibodies. Multiplex-PCR and oligonucleotide arrays were utilized for the study of herpesviral DNA. All of the samples, except one OND CSF sample that contained HSV-1 DNA, were negative for herpesviral DNA.

HHV-6A antibody titers were significantly higher in patients with MS. The mean titer of HHV-6A in patients with OND was 27 with a standard deviation (SD) of 31. In comparison, patients with CDMS had a mean titer of 87 with a SD of 101 ($p = 0.005$) and patients with CPMS had a mean titer of 118 with a SD of 92 ($p = 0.00002$). Initial HHV-6A studies in regard of prevalence and mean titers suggested an association between HHV-6A and multiple sclerosis, although HHV-6 antibody formation might be an epiphenomenon associated with autoimmune mechanisms and polyclonal B-cell activation. Indeed, elevated antibody titers to a number of infectious agents have been reported. Still, one of the strongest links between EBV and MS is an altered immune response to EBV in MS (Sundström *et al.*, 2004).

Avidity of HHV-6A and HHV-6B was investigated by elution of low-avidity IgG antibodies with urea. One of the patients with OND had low-avidity antibodies to HHV-6B in serum suggesting recent primary infection with HHV-6B. None of the patients with either CDMS or CPMS had low-avidity antibodies to HHV-6B. Two patients with CDMS and three patients with CPMS in comparison to one patient with OND had low-avidity antibodies to HHV-6A suggesting recent primary infection with HHV-6A. Although not a statistically significant finding, it seems that HHV-6A primary infection in patients with MS might be more common than in patients with other neurological diseases. Low-avidity antibodies are present in serum two to three months after primary infection, and after that B-cells have matured and high-avidity antibodies are produced. The time frame is quite narrow if related to the relatively slow progression and development of the chronic disease, like multiple sclerosis, and therefore it is possible that the number of primary HHV-6A infections among the patients with MS could be even higher, if looked at the right time. An interesting observation was that the largest proportion of subjects that had low-avidity antibodies were patients with possible MS. These patients possibly have MS, although it is not definitely diagnosed. Typical to these patients is that they are in the early phase of the disease, and therefore, presence of low-avidity antibodies might suggest a temporal association between the onset of the disease and HHV-6 infection.

To get further information on the role of HHV-6 in MS, corresponding CSF samples from the same patients were studied for HHV-6 IgG antibodies. All of the CSF samples, including patients with MS and OND, were negative for HHV-6B IgG. All of the patients with OND were also negative for HHV-6A IgG, but three patients with CDMS (11.1%, $p = 0.06$) and four patients with CPMS (21.1%, $p = 0.01$) had HHV-6A IgG in CSF. Either the antibodies are infiltrated from serum to CSF through broken blood brain barrier (BBB) or antibodies are produced within CNS as a result of viral CNS infection.

Intrathecal HHV-6 antibody production in MS (II)

Two patients with CDMS and three patients with CPMS had an abnormal AbI ratio suggestive of intrathecal antibody production. One patient with positive CSF finding in each patient group had unremarkable AbI probably suggesting infiltration of IgG from serum to the CSF. The breakdown of the BBB in MS might lead to increased transmission of serum molecules like immunoglobulins to the central nervous system and CSF. Thus it is important to compare CSF antibody findings to the corresponding serum antibodies and preferentially correct the results with total IgG content. Altogether two out of 27 (7.4%) of patients with CDMS and three out of 19 (15.8%) of patients with CPMS had intrathecal antibody production. Derfuss *et al*. (2005) found in their study that 8/38 (21.1%) of patients with definite MS and 4/13 (30.8%) of patients with possible MS had intrathecal antibodies. In their study the number of patients with intrathecal HHV-6 antibodies was even higher and supports together with our results that there is a subgroup of patients that have intrathecal immune response against HHV-6. Although Derfuss and others (Derfuss *et al.*, 2005) postulate in their article that their results excludes HHV-6 as a cause of MS in majority of patients (non-intrathecal HHV-6 antibodies), it is not excluded that HHV-6 acts as a trigger in early disease by a "hit and run" mechanism. They also suggest that specific intrathecal antibody production is either the consequence of more frequent reactivation of the virus or it could be part of polyspecific IgG production. We suggest a third option in which primary HHV-6A infection might act as a trigger and in some cases the infection invades CNS and becomes chronic. This might partially clarify our results that we found low-avidity antibodies more frequently from the patients with CPMS. It might be that activated B-cells producing HHV-6 antibodies have not reached the CNS yet. Interestingly, the two definite MS cases who had intrathecal antibodies were both patients with past HHV-6 infection suggesting chronic infection.

Intrathecal antibodies have been reported to be directed against other viruses and bacteria as well. These infectious agents include measles, rubella, VZV and Chlamydia pneu-

moniae (Sindic *et al.*, 1994; Luxton *et al.*, 1995; Reiber *et al.*, 1998; Derfuss *et al.*, 2001).

HHV-6 specific oligoclonal IgG bands in MS (III)

Nine patients with MS, four CDMS and five CPMS, collected from the initial patient population used in study II, were further analyzed for the presence of oligoclonal bands. The patients were included to the study using positive HHV-6 IgG CSF or low-avidity HHV-6 IgG antibodies in serum as inclusion criteria. All four patients with definite diagnosis had OCBs and one out of five patients with possible diagnosis had OCBs. The specificity of the bands to HHV-6 was then studied. In two patients with definite diagnosis the bands reacted with HHV-6 antigens, both HHV-6A and HHV-6B. These patients both had been serologically determined to have past HHV-6 infection. In two OCB-positives, HHV-6-OCB-negative, HHV-6 infection was serologically suggested to be an acute primary infection. Thus it might be that HHV-6 first infects peripheral compartments and invades CNS thereafter and generates a chronic CNS infection, after which oligoclonal HHV-6-specific antibodies are produced. We compared CNS-specific banding pattern from MS patient that reacted with HHV-6 to banding pattern seen in HHV-6 reactivation case. In the control case the banding pattern was clearly different, strong OCBs were seen in serum but only faint bands in CSF. This was clearly opposite to what we noted in MS. In these patients virus was clearly linked to the etiology of the disease. In addition, HHV-6-specific OCBs did not react with HSV-1, although a control case with possible HSV encephalitis reacted with HSV-1 antigen, showing no cross-reaction between these two viruses.

The presence of autoantibodies in MS is well characterized; however, the specificity of the autoantibodies is not well specified (Lovato et al., 2008). It is evident that some of the OCBs are directed against selfantigens including myelin components. Besides selfantigens, some infectious antigens including two EBV proteins (Cepok et al., 2005) and measles antigen (Vandvik et al., 1976) have been reported to react with OCBs.

Table 5. HHV-6 antibody findings in nine selected patients with MS

No	Diagnose	HHV-6 status	HHV-6 IgG CSF/ITAP	OCB/HHV-6 OCB
1.	CDMS	Past	+ + + / +	+ / +
2.	CDMS	Primary	-/-	+ /-
3.	CDMS	Past	+ + / +	+ / +
4.	CDMS	Primary	+ + /-	+ /-
5.	CPMS	Primary	+ / +	-/-
6.	CPMS	Primary	+ / +	-/-
7.	CPMS	Past	+ / +	-/-
8.	CPMS	Primary	-/-	-/-
9.	CPMS	Past	+ /-	+ /-

HHV-6A primary infection in children with neurological symptoms (IV)

Forty-one children of 53 (77%) with suspected encephalitis had HHV-6A antibodies. Six of them (Table 5) had low-avidity antibodies in their serum suggesting temporal relation with primary HHV-6 infection and neurological symptoms. The mean age of children with primary infection was significantly lower than that of others (2.3 vs. 6.9 yrs). Three children (Nos 1, 2, 3) had no other virological findings in extensive studies. Two of them (1, 2) also had evidence of HHV-6 DNA, one in serum and one in CSF; anyhow we were not able to identify the variant by PCR. The third child was negative for HHV-6 DNA in serum, and CSF sample was not available. One child (No 6) with low-avidity antibodies to both HHV-6A and HHV-6B had also HHV-6 DNA in CSF, but had also Coxsackie B5 in stool and antibodies to both adenovirus and Coxsackie B5 in CSF.

Table 6. HHV-6 DNA findings in children with low-avidity antibodies.

No	Low-avidity antibodies	Serum DNA	CSF DNA
1.	HHV-6A	-	(+)*, -
2.	HHV-6A	+	-
3.	HHV-6A	-	NA
4.	HHV-6A	-	NA
5.	HHV-6B	-	-
6.	HHV-6A&B	-	+

*positive on day 1, negative on day 15; NA, not available

Altogether 6/53 (11.3%) children with neurological symptoms had low-avidity antibodies in their serum. In three cases the presence of virus was confirmed by PCR, only one in serum. This is however, to be expected since DNA appears transiently in serum, usually before the appearance of antibodies. Unfortunately, CSF samples were not available from all of the patients.

Concluding remarks and perspectives

Human herpesvirus 6 (HHV-6) is a very neurotropic virus. It has been shown to be one of the most prominent infectious candidates to play a role in the pathogenesis of MS. However, the nature of the virus, its ubiquity, latency, viral DNA integration into host genome, as well as methodological differences have resulted in controversial reports on the association of HHV-6 and MS. Since it is obvious that conventional Koch's postulates, e.g. infectious agent must be found in every case and not present in any control case, do not apply in this situation, we need to devise new approaches to clarify the role of such infectious agents that do not cause disease in everyone. Koch's postulates are more applicable for bacteria than viruses. Attempts to create more applicable criteria for the disease causation have been developed for commensal viruses (for review see, Fredricks and Relman, 1996).

In our studies we were able to show an expression of HHV-6 antigen in MS brain autopsy samples. The expression of HHV-6 antigen was more frequent in MS cases than in controls. We also showed increased prevalence of HHV-6 antibodies both in serum as well as in CSF. The presence of IgG low-avidity antibodies to HHV-6 in serum suggested more recent acquisition of the virus. Interestingly, most cases with recent exposure to HHV-6 were possible MS cases. This might suggest that HHV-6 have a role at the early disease. Others have also reported that HHV-6 infection might be more common in early disease, by detection of HHV-6 DNA by PCR (Rotola et al., 2004) or by detection of HHV-6 IgM (Villoslada et al., 2003). In some MS cases antibodies were found in the CSF, no HHV-6 antibodies were found in CSF of patients with OND. Antibodies were shown to represent antibodies that are synthesized locally within the central nervous system suggesting a CNS infection.

We were able to identify OCBs in the CSF of two patients with MS that reacted with HHV-6 antigen. This suggests direct involvement of HHV-6 in the formation of OCBs, which are one of the hallmarks of MS and therefore suggests HHV-6 as a causal agent in these patients. This is a first time that specificity of HHV-6 to OCBs has been shown. Some EBV proteins, as well, have been shown to react with OCBs; however, we showed that HHV-6 viral antigen reacted with most if not all of the OCBs present in CSF. This implies that HHV-6 is a major antigenic force driving the immunological response and formation of OCBs in these patients. The EBV proteins that have been shown to react with OCBs are EBNA-1 and BRRF2 (Cepok et *al.*, 2005). BRRF2 is an uncharacterized protein and EBNA-1 is a latent protein. However, most of the studies, including ours (I), have been unable to show EBV within CNS.

Studies comprising this thesis supported the association of HHV-6 and MS in some cases. However, it seems probable that there is no single infectious cause for MS; instead many different pathogens might act as triggers in the development of the disease. The strongest candidates thus far are HHV-6 and EBV.

HHV-6 is predominantly regarded as a childhood virus that causes ES. Besides ES, primary infection may cause febrile seizures and infections of the CNS. We studied the presence of low-avidity IgG antibodies in children with suspected encephalitis. We found a remarkable number of children who had low-avidity IgG antibodies to HHV-6 concomitantly with the signs or symptoms of encephalitis suggesting a significant involvement of HHV-6 as a causal agent of encephalitis at young ages. HHV-6 might be a underappreciated cause of encephalitis both in immunocompromised as well as in immunocompetent individuals. It is important to test for HHV-6 in patients with encephalitis of unknown etiology.

Carefully designed clinical trials using efficient antiviral drugs to HHV-6 in selected cases of MS, as well as with encephalitis, are urgently needed for the clarification of the role of HHV-6 in MS and encephalitis. In case studies,

antiviral drugs like ganciclovir, have been successfully used for the treatment of HHV-6 encephalitis. Unfortunately, there have been no well-defined clinical trials for efficacy of antiviral drugs in the treatment of HHV-6 infection. The drugs used currently for the treatment are drugs that are used for the treatment of CMV. Both *in vitro* experiments to seek for new antiviral compounds and carefully designed clinical trials are needed to establish effective treatments for HHV-6 infection. Furthermore, special attention should be given to the development of suitable animal models for the study of HHV-6 pathogenesis. The finding of HHV-6 specific OCBs in MS is of special interest and needs to be further confirmed and evaluated in larger patient populations.

Acknowledgments

This study was carried out at the Department of Virology, Haartman Institute, University of Helsinki. The former and present heads of the department, professors Antti Vaheri, Klaus Hedman and Kalle Saksela are acknowledged for providing such an inspiring research facilities.

I would like to express my sincere gratitude to my supervisors docent Marjaleena Koskiniemi and professor Antti Vaheri. Marjaleena's clinical point of view to research together with Antti's outstanding general knowledge on research and science has given a firm foundation to grow as a scientist.

Reviewers of this thesis, Irina Elovaara, MD, PhD and Veijo Hukkanen, MD, PhD, are greatly acknowledged for invaluable comments that improved the manuscript. Associate professor Malcolm Richardson is acknowledged for language revisions.

I am grateful to Markus Färkkilä, MD, PhD, the Head of the Department of Neurology, for pre-screening patients together with Juha Multanen, MD. Staff of the Neurological Outpatient Clinic at HYKS are thanked for collecting samples. I owe my gratitude to Lasse Uotila, MD, PhD from the Department of Clinical Chemistry, Protein Chemistry Lab and all the people from the lab for helping me with the IEF.

Sincere thanks to all my co-writers in original publications.

I thank the people from the former PA group, Heli, Irina, Josef, Miina, Tambet and Vappu. "Fenix group", Nemosis Research Group, which rose from the ashes of the PA group; Anna, Kati and Perttu are thanked for sharing coffee and ideas on Tuesday PA-meetings. Wei Wei and Walter are also thanked for friendship. I am especially grateful to Wei and Irina for introducing me to the fascinating world of laboratory experiments.

It has been refreshing to have scientific as well as "nothing-to-do-with-science" discussions with Anna, Anne, Anu, Jussi, Kati, Matte, Perttu, Tarja,

Tomas, Walter as well as with other graduate students at the Department of Virology.

Docent Irmeli Lautenschlager, Dr. Raisa Loginov and Laura Mannonen, MSc, are thanked for discussions on HHV-6 and for attending together with me to HHV-6/7 meetings in Barcelona and Baltimore. Docent Maija Lappalainen and Dr. Suvi Bühler are thanked for discussion and for their interest to my research as well as providing samples.

Thanks to monkeys: Kettu, Marco, Boris, Mr. Van, Kungen, Jones, Rebound, Mini, Sakke, GM (a.k.a. Kives), Are, Federer, Jääpallomies, Nuti, Härkä, Benkku alias Taikuri, Koutsi, Kibe. We ROCK.

I thank all the players participating Monday-sähly. It's always a great start to the week.

Every time that we have made the World better place to live with Heikki & Jenni, Jaana & Juha, Jani & Maiju, Jukka & Elina, Marco & Jarkko, Mika & Niina, Sakari & Anna and Tomi & Tuija, I have had a terrible thirst at next morning (Did you know that having a hangover is not having enough water in your body to run your Krebs cycle? Which is exactly what happens to you when you're dying of thirst. So, dying of thirst would probably feel pretty much like the hangover that finally bloody kills you. – Charles Herman (the Beautiful Mind (2001)). Thanks for those parties and get-togethers to all you. I would like to thank Sakke, Puhis, Jani for all those fishing trips to Finland/Norway/Sweden. Special thanks to Marco for helping me with the lay-out of this thesis.

My parents Pia and Kosti are acknowledged for their support in everything that I have ever decided to do. Thanks to my grandma Maj-Lis. Thanks to my brother Ippe and his family Hanna, Pihla and Pilvi(?) and to my parents-in-law Jorma and Marjukka and to sister-in-law Sini and her family Tero and Riina. Finally, warm thanks to my wife Inka for her love and support.

The Finnish Cultural Foundation, the Helsinki Biomedicum Foundation, the Helsinki University Research Fund, the Helsinki University Central Hospital Research and Education Fund and the HHV-6 Foundation are thanked for financial support.

Helsinki, December 2008

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